# UNRAVELLING CITRUS HUANGLONGBING DISEASE

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PUBLISHED IN: Frontiers in Plant Science

frontiers Research Topics

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ISSN 1664-8714
ISBN 978-2-88966-341-5
DOI 10.3389/978-2-88966-341-5

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# UNRAVELLING CITRUS HUANGLONGBING DISEASE

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**Citation:** Ferrarezi, R. S., Urbaneja, A., Machado, M. A., Vincent, C. I., eds. (2021). Unravelling Citrus Huanglongbing Disease. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88966-341-5

# Table of Contents

frontiers in Plant Science

EDITORIAL
published: 13 November 2020
doi: 10.3389/fpls.2020.609655

# Editorial: Unravelling Citrus Huanglongbing Disease

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**Keywords: *Candidatus* Liberibacter spp., disease management, infection, citriculture, vector management, citrus under protected screen**

## Editorial on the Research Topic

## Unravelling Citrus Huanglongbing Disease

Huanglongbing (HLB) or citrus greening is a disease caused by the unculturable, fastidious, phloem-restrictive, Gram-negative bacterium *Candidatus* Liberibacter spp. Currently, there are three species linked to the disease. The Asian form associated with *Candidatus* Liberibacter asiaticus (CLas) is heat-tolerant and can survive well above 30°C. The African (*Candidatus* Liberibacter africanus) and American forms are heat-sensitive and develop between 22 and 25°C (*Candidatus* Liberibacter americanus) (Bové, 2006). Huanglongbing is vector-transmitted mainly by the African citrus psyllid *Trioza erytreae* Del Guercio (Hemiptera: Triozidae) and the Asian citrus psyllid (ACP) *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), with two other psyllids also reported as vectors, *D. communis* Mathur and *Cacopsilla citrisuga* (Yang & Li) (Hemiptera: Pysllidae). The disease was first described in 1929 and reported in China in 1943. The African variation was reported in South Africa in 1947. The disease was reported in Brazil (São Paulo) in 2004 and the United States (Florida) in 2005. More than 20% of citrus trees in Brazil and 90% in Florida are currently affected, with symptomatic trees present in Texas and California. Huanglongbing is present and affects several citrus-producing countries of Asia, sub-Saharan Africa, and America (except for Bolivia, Chile, Perú, and Uruguay). The Mediterranean Basin and Australia are still free of HLB. The threat to HLB-free countries is constant due to the proximity of the disease and its vectors and the unstoppable increase in international trade.

*Candidatus* Liberibacter asiaticus can infect most citrus species, cultivars, and hybrids. Leaves of infected trees develop a blotchy mottle symptom (yellowing vein and an asymmetrical chlorosis). Infected branches suffer substantial leaf drop, resulting in severe canopy thinning. Fibrous root density decreases nearly 30%, directly affecting water and nutrient uptake, severely reducing fruit yield, and demanding more frequent irrigation and improved mineral nutrition practices. Fruit from HLB-affected trees are often lopsided, poorly colored, and contain aborted seeds, with low commercial value due to small size and quality. The juice from affected fruit present low soluble solids content, high acidity, and bitterness.

There is no cure for the disease yet. Current management strategies focus on either delaying infection or managing infected trees. Methods of delaying infection include removal of symptomatic trees, planting and resetting using HLB-free nursery trees, protection of grove edges and intensive monitoring and control of the vectors mainly using physical, chemical, and biological control methods. Management of infected trees includes adjusting soil pH, enhancing nutritional programs, and improving irrigation management based on altered tree capacities and needs when



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***Specialty section:***
*This article was submitted to Plant Pathogen Interactions, a section of the journal Frontiers in Plant Science*

***Received:*** *23 September 2020*
***Accepted:*** *02 October 2020*
***Published:*** *13 November 2020*

***Citation:***
*Ferrarezi RS, Vincent CI, Urbaneja A and Machado MA (2020) Editorial: Unravelling Citrus Huanglongbing Disease. Front. Plant Sci. 11:609655. doi: 10.3389/fpls.2020.609655*

affected by HLB. Research has evolved rapidly to address this devastating challenging, and several recent alternatives based on psyllid management, bactericides, cultural practices (thermotherapy and vector exclusion using netting), and genetic transformation have been tested. While most attempts at management have focused on a single component of the disease pyramid, most do not explicitly consider multiple elements at the same time.

This Research Topic is a collection of 9 articles from 49 co-authors and present the latest advances in managing the HLB pathosystem, focusing on assessments of near-term feasible practices in the context of the vector, pathogen, host plant, and environment.

## VECTOR

Britt et al. identified possible biological control candidates by conducting one of the most comprehensive surveys of natural ACP populations in major citrus production regions spanning 21 counties in Florida. By optimizing PCRs and RT-PCRs, authors successfully detected and monitored the prevalence of five previously identified ACP-associated RNA and DNA viruses throughout Florida citrus groves, which include: *D. citri*-associated C virus, *D. citri* flavi-like virus, *D. citri* densovirus, *D. citri* reovirus, and *D. citri* picorna-like virus. High-throughput sequencing generated data also revealed that the most abundant virus in Florida ACP populations was citrus tristeza virus (CTV), which is not an ACP-associated virus, suggesting persistent presence of CTV infection in citrus throughout Florida groves. Collectively, information obtained from the study may help guide the direction of biotechnological pest control efforts involving several viruses that were detected for the first time in Florida ACP populations, including two newly identified ACP-associated viruses.

Khan et al. evaluated the brown lacewing *Sympherobius barberi* as a biological control agent against the *D. citri* and frozen eggs of the Mediterranean flour moth *Ephestia kuehniella*. Adult *S. barberi* successfully fed on *D. citri* eggs and nymphs under both light and dark conditions. The Asian Citrus Psyllid was also suitable for the development and reproduction of *S. barberi* except for slightly prolonged larval development compared with *E. kuehniella* diet. The egg hatch from the total number of eggs laid on *D. citri* and *E. kuehniella* diets averaged 65 and 52%, respectively. Females laid 64% eggs on dimpled white paper compared to 36% combined on plain paper and leaves of citrus, orange jasmine, eggplant and cantaloupe. *Sympherobius barberi* released at densities of 2–6 adults against eggs and nymphs of *D. citri* on infested orange jasmine in cages provided a reduction of 43–81% in the number of eggs or nymphs. In the field tests on *D. citri* infested citrus trees, there was a 35% reduction in five cohorts in which developing colonies of 28–32 nymphs were provided to one *S. barberi* per cage. The findings suggest the potential of *S. barberi* as a predator of *D. citri* and to contribute to reducing HLB.

Patt et al. determined whether synthetic compounds, which were ligands of *D. citri* olfactory binding protein DCSAP4, influenced the settling and aggregation levels of psyllids on young citrus shoots. Authors found the test ligands themselves did not attract the psyllids but rather modulated the psyllid's response to a mixture of citrus volatiles. The results suggest that synthetic ligands of *D. citri* chemosensory binding proteins can be used to increase the effectiveness of citrus scent lures used to attract psyllids to monitoring traps and attract and kill devices.

## PATHOGEN

Vincent et al. assessed two grower-used therapies, heat treatment and foliar anti-bacterial application, following an industry claim that heat treatment improved subsequent systemic uptake of foliar-applied anti-bacterial compounds. Heat treatment and defoliation treatments reduced growth but did not affect systemic delivery of oxytetracycline. Oxytetracycline was detected in nearly all covered leaf samples in both repetitions, though at lower concentrations than in directly applied leaves. Authors concluded that neither heat treatment nor leaf age strongly affect systemic oxytetracycline delivery, and further discussed implications of the study for leaf age effects on foliar delivery and for phloem delivery of foreign compounds through foliar application.

Zhang et al. implemented an integrated strategy that includes chemotherapy, thermotherapy, and additional nutrition treatment in three different field trials over 3 consecutive years. Analysis showed that most of the tested chemicals were effective to some degree in killing or suppressing the bacterium, with higher therapeutic efficacy. Trunk-injected penicillin G potassium was the most effective chemical treatment in all groves, followed by oxytetracycline, and silver nitrate delivered as foliar sprays. Although the steam heat treatment and additional nutrition did not eliminate or suppress HLB over the long term, these treatments did positively affect tree growth and recovery in the short term. Overall, the results provided new insights into HLB control method and strategy for integrated management for HLB epidemic groves.

## HOST PLANT

Deng et al. examined transverse sections of leaf lamina and midribs with light and epifluorescence microscopy to determine anatomical characteristics that underlie HLB-tolerant mechanisms operating among "Bearss" lemon, "LB8-9" Sugar Belle® mandarin and its sibling trees compared with HLB-sensitive "Valencia" sweet orange. Although there were physical, morphological, and pathological similarities in the examined foliage, internal structural preservation in lemon and mandarin was superior compared with HLB-sensitive sweet orange and siblings of mandarin. Intriguingly, there was substantial phloem regeneration in the tolerant types that may compensate for the dysfunctional phloem, in comparison with the sensitive selections. Authors attribute the lower levels of phloem disruption and greater phloem regeneration as two key elements that contribute to HLB tolerance in diverse citrus cultivars.

Huang et al. genotyped an intergeneric F1 population of sweet orange and trifoliate orange by genotyping-by-sequencing and constructed high-density SNP-based genetic maps separately for trifoliate orange and sweet orange. Progenies of the F1 population and their parents were planted in a replicated field trial, exposed to intense HLB pressure for 3 years, and then evaluated for susceptibility to HLB over 2 years. Most of the identified QTLs each explained 18–30% of the phenotypic variation, indicating their major role in determining HLB responses. These results show, for the first time, a quantitative genetic nature as well as the presence of major loci for the HLB tolerance in trifoliate orange. The results suggest that sweet orange also contains useful genetic factors for improving HLB tolerance in commercial citrus varieties. Findings are valuable and timely for genetic solutions to the devastating HLB crisis through breeding, genetic engineering, or genome editing.

## ENVIRONMENT

Ferrarezi et al. assessed the ability of large-scale enclosed screenhouses to exclude the ACP, stop HLB inoculation and dissemination, and improve fruit yield of in-ground and container-grown 6-year-old "Ray Ruby" grapefruit at super-high planting densities relative to open-air trees. Despite the weather-related damages to the screens, only trees cultivated in open-air tested positive for *C*Las after 6 years. There was fast disease progression for all outside treatments, with 100% infection. The screenhouses provided disease exclusion, increased fruit yield and fruit quality, representing an alternative for growers interested in producing high-quality fruit for the fresh market.

Dala-Paula et al. provided a review analyzing and discussing the effects of HLB on orange juice quality to help the citrus industry manage the quality of orange juice and guide future research needs. Symptomatic oranges show higher titratable acidity and lower soluble solids, solids/acids ratio, total sugars, and malic acid levels. Among flavor volatiles, ethyl butanoate, valencene, decanal and other ethyl esters are lower, but many monoterpenes are higher in symptomatic fruit compared to healthy and asymptomatic fruit. The disease also causes an increase in secondary metabolites in the orange peel and pulp, including hydroxycinnamic acids, limonin, nomilin, narirutin, and hesperidin. Resulting from these chemical changes, juice made from symptomatic fruit is described as distinctly bitter, sour, salty/umami, metallic or musty and lacking in sweetness and fruity/orange flavor. Those effects are reported in both "Valencia" and "Hamlin" sweet oranges, two cultivars that are commercially processed for juice in Florida. Earlier research showed that HLB-induced off-flavor was not detectable in juice made with up to 25% symptomatic fruit in healthy juice, by chemical or sensory analysis. However, a blend with a higher proportion of symptomatic juice would present a detectable and recognizable off flavor.

## OUTLOOK AND FUTURE CHALLENGES

Huanglongbing will continue to be the main citrus disease in the world. In recent years, there has been a significant increase in the volume of scientific information on all aspects of citrus relations with the bacteria, the vector, and the environment. Despite all this information, the scientific community has not yet managed to bring a definitive solution to the control of the disease. The disease is highly complex, affecting the transport system leading to phloem transport and carbohydrate distribution dysfunction.

The special edition of Frontiers dedicated to HLB was an attempt to expand the forum for the presentation of new results and to contribute with new approaches to the understanding of the disease and its control. Publishers hope that these opportunities will continue and expand. Only the contribution of the scientific and technological community can bring a definitive solution to the main challenges of citrus production worldwide.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

## FUNDING

Funding for this research was provided by the U. S. Department of Agriculture's Specialty Crop Research Initiative (SCRI) Citrus Disease Research and Extension Program (CDRE) (Award #2018-70016-27387).

## ACKNOWLEDGMENTS

We greatly appreciate the efforts of the journal editors and staff, peer reviewers, and authors.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 27 November 2018
doi: 10.3389/fpls.2018.01694

# Construction of High-Density Genetic Maps and Detection of QTLs Associated With Huanglongbing Tolerance in Citrus

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OPEN ACCESS

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**Reviewed by:**
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**Specialty section:**
*This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Plant Science*

**Received:** *31 July 2018*
**Accepted:** *31 October 2018*
**Published:** *27 November 2018*

**Citation:**
*Huang M, Roose ML, Yu Q, Du D, Yu Y, Zhang Y, Deng Z, Stover E and Gmitter FG Jr (2018) Construction of High-Density Genetic Maps and Detection of QTLs Associated With Huanglongbing Tolerance in Citrus. Front. Plant Sci. 9:1694. doi: 10.3389/fpls.2018.01694*

Huanglongbing (HLB), or citrus greening, is the most devastating disease in citrus worldwide. Commercial citrus varieties including sweet orange (*Citrus sinensis*) are highly susceptible to HLB, and trifoliate orange (*Poncirus trifoliata*, a close *Citrus* relative) is widely considered resistant or highly tolerant to HLB. In this study, an intergeneric $F_1$ population of sweet orange and trifoliate orange was genotyped by Genotyping-by-Sequencing, and high-density SNP-based genetic maps were constructed separately for trifoliate orange and sweet orange. The two genetic maps exhibited high synteny and high coverage of the citrus genome. Progenies of the $F_1$ population and their parents were planted in a replicated field trial, exposed to intense HLB pressure for 3 years, and then evaluated for susceptibility to HLB over 2 years. The $F_1$ population exhibited a wide range in severity of HLB foliar symptom and canopy damage. Genome-wide QTL analysis based on the phenotypic data of foliar symptom and canopy damage in 2 years identified three clusters of repeatable QTLs in trifoliate orange linkage groups LG-t6, LG-t8 and LG-t9. Co-localization of QTLs for two traits was observed within all three regions. Additionally, one cluster of QTLs in sweet orange (linkage group LG-s7) was also detected. The majority of the identified QTLs each explained 18–30% of the phenotypic variation, indicating their major role in determining HLB responses. These results show, for the first time, a quantitative genetic nature yet the presence of major loci for the HLB tolerance in trifoliate orange. The results suggest that sweet orange also contains useful genetic factor(s) for improving HLB tolerance in commercial citrus varieties. Findings from this study should be very valuable and timely to researchers worldwide as they are hastily searching for genetic solutions to the devastating HLB crisis through breeding, genetic engineering, or genome editing.

**Keywords: *Candidatus* Liberibacter, genetic map, $F_1$ population, genotyping-by-sequencing, *Poncirus*, QTL mapping, SNP**

## INTRODUCTION

Huanglongbing (HLB), commonly known as citrus greening, is the most devastating disease in citrus plantations worldwide. Since the first identification of the disease in Florida in 2005, HLB has spread throughout the state, and is now found in most states in the United States where citrus is grown. It is rapidly spreading throughout many of the world's production areas, as well. Due to widespread infection and lack of effective management strategy, Florida's nearly $11 billion citrus industry has experienced a rapid and continuous decline. The production of sweet orange dropped from 240 million boxes in 2004 to 45 million in 2018 (Florida Citrus Commission[1]).

Huanglongbing-diseased trees develop leaves with characteristic blotchy mottle, the shoots are stunted and yellowing, and the canopy and branches gradually dieback as the disease progresses. These symptoms are associated with the host limitations for photoassimilate transport and nutrient uptake induced by the disease, and finally can lead to tree death (Blaustein et al., 2018). HLB is generally considered to be caused by three species of *Candidatus* Liberibacter, of which *Candidatus* Liberibacter asiaticus (*C*Las) is the most widespread and virulent species and is the only species reported in the citrus industry of United States. *C*Las is a heat-tolerant Gram-negative bacterium, resides only in the phloem of plant hosts, and is vectored by the sap-sucking Asian citrus psyllid (ACP, *Diaphorina citri*) (Wang N.A. et al., 2017). As an obligate and insect-transmitted plant pathogen, *C*Las attacks all species and hybrids in the genus of *Citrus* as well as some closely related genera (Ramadugu et al., 2016). Most commercial citrus cultivars are highly susceptible to HLB (Stover et al., 2016a,b). Within cultivated citrus, high levels of tolerance to HLB were mostly found in some types that are commonly used as rootstocks, such as trifoliate orange and some of its hybrids (Albrecht and Bowman, 2011, 2012; Ramadugu et al., 2016).

Until now, very little is known about the molecular mechanism of pathogenesis of *C*Las (Martinelli and Dandekar, 2017). As to understanding the genetic architecture of citrus resistance or susceptibility to HLB, there has been no compelling progress, and no QTL related to *C*Las infection or HLB tolerance responses has been reported. Moreover, so far there is no sustainable management and control of HLB where it is endemic. Breeding and development of HLB resistant or tolerant cultivars is widely regarded to be the most practical strategy to support long-term control of this severe disease in the field. The reports of variability for tolerance of or sensitivity to HLB within citrus and its relatives encourages breeding and selection for tolerant or possibly resistant genotypes (Richardson and Hall, 2013; Ramadugu et al., 2016; Killiny et al., 2017; Miles et al., 2017). Traditional breeding through crossing elite cultivars with resistant materials can achieve this objective. However, the introgression of resistant germplasm into elite cultivars will likely require multiple rounds of backcrossing to recover desirable commercial traits. In addition, the breeding cycle for citrus ranges from 5 to 10 years, and the rescue of the citrus industries through HLB-resistant or tolerant cultivars demands considerable urgency. Identification of QTLs associated with resistance or tolerance to HLB in citrus can facilitate more rapid development of resistant cultivars through marker-assisted selection or genome editing.

To identify QTLs associated with phenotypic traits, a genetic map with high resolution and fine accuracy is crucial. Nowadays, the wide application of high-throughput genome sequencing and efficient SNP genotyping allows the construction of genetic maps with numerous markers at an acceptable cost (Deschamps et al., 2012). While initially confined to annual herbaceous plants, high-density genetic mapping is increasingly extended to perennial woody plants. Using high-throughput genotyping, the saturation of genetic maps has been greatly improved for some citrus species, such as sweet orange, mandarin and pummelo (Lyon, 2008; Ollitrault et al., 2012a; Shimada et al., 2014; Guo et al., 2015; Yu et al., 2016; Curtolo et al., 2017; Imai et al., 2017). However, in comparison with well-studied model and agronomic plants, citrus lags in development of high-density, high-resolution genetic maps with fine accuracy and precision. Moreover, so far there is no high-density genetic map for trifoliate orange.

This study inaugurates investigation of HLB infection in citrus by repeated phenotyping of segregating populations and QTL mapping. The objectives of this study were: (1) to evaluate severity level of HLB disease among a field population exposed to intense HLB pressure using two phenotypic traits, foliar symptoms and canopy damage; (2) to construct high-density genetic maps separately for trifoliate orange and sweet orange in an $F_1$ mapping population through Genotyping-by-Sequencing; (3) to identify QTLs associated with citrus HLB infection and responses separately in trifoliate orange and sweet orange genetic maps.

## MATERIALS AND METHODS

### Plant Materials

Genotyping was carried out in an $F_1$ population of 170 individuals derived from mixed intergeneric crosses between two sweet oranges (*Citrus sinensis* 'Sanford' and 'Succari') and two trifoliate oranges (*Poncirus trifoliata* 'Argentina' and 'Flying Dragon'), including 79 progenies of 'Sanford' × 'Argentina', 40 progenies of 'Succari' × 'Flying Dragon', and 51 progenies of 'Flying Dragon' × 'Succari'. Of the genotyping population, 86 individuals were randomly chosen as a phenotyping population, including 47 progenies of 'Sanford' × 'Argentina,' 14 progenies of 'Succari' × 'Flying Dragon', and 25 progenies of 'Flying Dragon' × 'Succari'. In addition, two sweet orange varieties ('Navel' grafted on 'Swingle' and 'Hamlin' grafted on 'C-35'), six trifoliate orange varieties ('Flying-Dragon', 'Argentina', and 'Pomeroy' grafted on 'Volkamer' lemon; and seedlings of 'Rubidoux', 'Rich 16-6', and 'Large-flower'), and seedlings of 'Volkamer' lemon (*Citrus volkameriana*) were included as controls in the phenotyping. All progenies were clonally propagated by grafting on 'Volkamer' lemon in the greenhouse of the USDA/ARS in Fort Pierce, FL in 2010. Except for 'Volkamer' lemon seedings that had

[1]http://www.floridacitrus.org

16 replicate trees, each of the progenies and control varieties had eight clonal replicate trees. A completely randomized experiment of 768 trees was established in a field trial at the USDA-ARS in Fort Pierce in 2011. The planting consisted of eight rows oriented south-north, with 4 m × 1.5 m planting distances. Guard citrus trees not analyzed in this study were planted at the ends of each row. The field population trees were irrigated and fertilized with professional practices, but pesticides were not applied during the study, to encourage psyllid population increase, feeding, colonization, and inoculation of *C*Las to the trees. Maintenance of the field trees was as described for a nearby experiment on the same farm (Richardson et al., 2011).

## Detection of *C*Las Infection

As reported in other concurrent studies (Lewis-Rosenblum et al., 2015; Ramadugu et al., 2016), the HLB disease pressure was high in the field trial at this location, which caused homogeneous inoculation of *C*Las naturally, providing excellent conditions to evaluate trees under natural disease spread conditions (Westbrook et al., 2011; Richardson and Hall, 2013). During the period of HLB disease evaluation, the status of *C*Las infection for each tree was diagnosed using the TaqMan label-based multiplex real-time PCR method (Li W.B. et al., 2006, 2008). At least four fully expanded mature leaves were randomly collected from different branches and different quadrants of each tree. The midribs were separated from the leaves and cut into small pieces for DNA extraction using the CTAB method (Aldrich and Cullis, 1993). Real-time qPCR was performed on an ABI 7500 thermocycler with probes specific to *C*Las 16S ribosomal gene and citrus cytochrome oxidase gene. The mean cycle threshold (C*t*) values of qPCR for direct *C*Las detection were normalized with C*t* values of the corresponding host plant gene. Trees with C*t* value under 33 were considered to be HLB-positive (Albrecht and Bowman, 2012).

## Evaluation of HLB Disease

Evaluations of HLB disease symptoms were performed twice per year from 2015 to 2016 in September and October, which is the optimal time for HLB disease evaluation considering both the growth condition of citrus (*Poncirus* and some of its hybrids are deciduous or semi-deciduous) and the period of the most evident disease symptoms. At each time of disease evaluation, the visual evaluation of foliar symptom and canopy damage was conducted twice per tree on each side along the tree rows. All evaluations were carried out by the same individual researcher. Foliar disease symptom severity was assessed on a 6-point scale visual rating for comprehensive typical HLB symptom including mottled rugose leaves and yellow shoots: 0 = no symptom for whole tree, 1 = isolated (less than 1/10 of tree) slight symptom, 2 = partly (approximately 1/3 of tree) slight symptom or isolated severe symptom, 3 = mostly (approximately 2/3 of tree) slight symptom or partly severe symptom, 4 = mostly severe symptom, 5 = severe symptom for whole tree. The slight foliar symptom rating refers to trees with leaves that are slightly mottled, pale or leathery, while the severe symptom rating refers to severely blotchy mottled, rugose or leathery. Canopy damage severity was also assessed on a 6-point scale visual rating basing on dieback, defoliation or stunting: 0 = full tree canopy without apparent dieback or defoliation or stunting, 1 = full tree canopy with isolated short dieback or very slight defoliation, 2 = partly short dieback or isolated long dieback or slight defoliation or stunting, 3 = mostly short dieback or partly long dieback or moderate defoliation or stunting, 4 = mostly long dieback or main branch dieback or severe defoliation or stunting, 5 = trunk dieback or whole tree dead. The mean rating score for each genotype in each year was the averaged rating score of all replicate trees at the two times of evaluation.

## Phenotypic Analysis

Descriptive statistics of all phenotypic data were calculated using IBM SPSS (Statistical Package for Social Sciences) Statistics 17.0. Significant differences between phenotypic data were declared with $p \leq 0.05$ by Student's *t*-test. To evaluate whether the data followed a normal distribution, a normality analysis by Kolmogorov–Smirnov and Shapiro–Wilk tests was performed separately for the dataset of each trait in each year. The Box-Cox transformation was performed before QTL analysis if data presented a non-normal distribution. Histograms for each trait were constructed using the mean rating score of the complete dataset. Pearson's correlation coefficients were calculated between year-means for each phenotypic trait.

## Genotyping by Sequencing

Genomic DNA of each citrange hybrid and parent was extracted using a modified CTAB method (Aldrich and Cullis, 1993), Genomic DNA was digested with PstI restriction endonuclease and then processed into restriction site associated DNA (RAD) libraries according to a previously described protocol (Baird et al., 2008). The constructed RAD libraries were sequenced on an Illumina HiSeq2000 platform following the manufacturer's protocol. Library construction, sequencing and SNP calling were provided by Floragenex (Floragenex Inc., Portland, OR, United States). SNP calling of parents and progenies was performed using the Clementine mandarin genome v1.0[2] as a reference. Genotypes at each locus were determined using the VCF Popgen Pipeline version 4.0 to generate a customized VCF 4.1 (variant call format) database with parameters set as follows: minimum allele frequency for genotyping 0.075, minimum Phred score 15, minimum depth of sequencing coverage of 12, and minimum 75% of individuals with the specific genotype call. The marker configuration codes "lm × ll" and "nn × np" were used to represent markers that were heterozygous only in one of the parents, and code "hk × hk" to represent markers that heterozygous in both parents.

## Linkage Analysis and Construction of Parental Linkage Maps

Due to asexual means of evolution (somatic mutations), molecular polymorphism among sweet orange cultivars is very rare (Ollitrault et al., 2012b); likewise very low polymorphism was found between the trifoliate orange varieties, so all $F_1$

[2]https://phytozome.jgi.doe.gov

progenies from different crosses between the two genera were considered as a single family, after excluding the inconsistent marker loci between parental varieties. To ensure high quality of linkage mapping for each parent, SNP markers segregating from only one of the parents (with configuration codes "lm × ll" and "nn × np") were selected to construct the linkage maps; markers with code "hk × hk" were not considered in this study. SNP markers matching the following criteria were excluded from linkage analysis: (1) had a missing genotype in more than 10% of progenies; (2) had a missing genotype for one of the parents; (3) had inconsistent genotypes among different parental sweet oranges or trifoliate oranges; (4) had homozygous genotypes for both parents; (5) had heterozygous genotype for both parents; (6) had genotypes in $F_1$ progeny not expected for the parental genotypes; (7) had no segregation in $F_1$ progeny. Segregation distortion was tested by $\chi^2$ conformity tests against the Mendelian segregation ratio of 1:1. Because the method of linkage analysis was based upon a test for independence of logarithm of the odds (LOD) scores that is not affected by segregation distortion, markers with certain skewed segregation were included in linkage analysis referring to previous studies on citrus (Bernet et al., 2010; Ollitrault et al., 2012a; Raga et al., 2012). Linkage analyses were performed using JoinMap 4.1 (Van Ooijen, 2011). Linkage mapping was performed under a two-way pseudo-testcross scheme (Grattapaglia and Sederoff, 1994) with two separate datasets, one with markers segregating from the trifoliate orange and the other one with markers segregating from sweet orange. Markers with identical segregation patterns or segregating similarity higher than 98% were excluded from the linkage groups. Phases of the linked marker loci were automatically detected by the software. Linked markers were grouped using the independence LOD with a threshold LOD score of 4.0 and a maximum recombination fraction (θ) of 0.4. Map distances were estimated in centiMorgans (cM) using the regression mapping algorithm and the Kosambi mapping function. The linkage groups were numbered according to the corresponding scaffold number of the reference Clementine mandarin genome, and marker names were composed of the scaffold number and SNP position on the reference genome. The genetic map was drawn using the MapChart 2.2 program (Voorrips, 2002).

## QTL Mapping

QTL mapping was carried out on two parental maps separately using MapQTL 6 under the backcross model by composite interval mapping (CIM) (van Ooijen, 1992). Phenotypic data were analyzed separately for each trait in each year. The LOD thresholds to declare a significant QTL for each trait were determined via permutation tests using 1,000 permutations at a genome-wide significance level of 0.90. The CIM analysis was performed in 1 cM steps to detect significant QTLs with a LOD score higher than the threshold. The nearest marker to the likelihood peak of each significant QTL was selected as a cofactor to perform restricted multiple QTL mapping (rMQM). If the LOD value linked with a cofactor fell below the threshold during rMQM mapping, the cofactor was removed, and the analysis repeated. This process was continued until the cofactor

**TABLE 1** | Summary of the genetic linkage maps of trifoliate orange and sweet orange.

| Linkage group | | Reference genome | Number of marker loci | | LG length (cM) | | Average inter-locus distance (cM) | | Number of syntenic markers | |
|---|---|---|---|---|---|---|---|---|---|---|
| Trifoliate orange | Sweet orange | Clementine mandarin | Trifoliate orange | Sweet orange | Trifoliate orange | Sweet orange | Trifoliate orange | Sweet orange | Trifoliate orange | Sweet orange |
| LG-t1 | LG-s1 | Scaffold_1 | 70 | 57 | 117.9 | 93.4 | 1.68 | 1.64 | 70 | 57 |
| LG-t2 | LG-s2 | Scaffold_2 | 87 | 89 | 124.5 | 99.6 | 1.43 | 1.12 | 87 | 89 |
| LG-t3 | LG-s3 | Scaffold_3 | 125 | 118 | 155.2 | 105.3 | 1.24 | 0.89 | 125 | 118 |
| LG-t4 | LG-s4 | Scaffold_4 | 64 | 94 | 121.5 | 74.0 | 1.90 | 0.79 | 64 | 88 |
| LG-t5 | LG-s5 | Scaffold_5 | 72 | 98 | 123.9 | 90.9 | 1.72 | 0.93 | 72 | 98 |
| LG-t6 | LG-s6 | Scaffold_6 | 52 | 77 | 78.7 | 68.2 | 1.51 | 0.89 | 52 | 77 |
| LG-t7 | LG-s7 | Scaffold_7 | 57 | 85 | 108.3 | 86.2 | 1.90 | 1.01 | 47 | 73 |
| LG-t8 | LG-s8 | Scaffold_8 | 58 | 76 | 102.8 | 82.1 | 1.77 | 1.08 | 58 | 64 |
| LG-t9 | LG-s9 | Scaffold_9 | 62 | 60 | 98.0 | 60.5 | 1.58 | 1.01 | 62 | 60 |
| | | Total | 647 | 754 | 1030.8 | 760.2 | 1.59 | 1.01 | 637 | 724 |

*The correspondence between linkage groups and syntenic scaffolds of Clementine mandarin genome was based on the location of most mapped markers.*

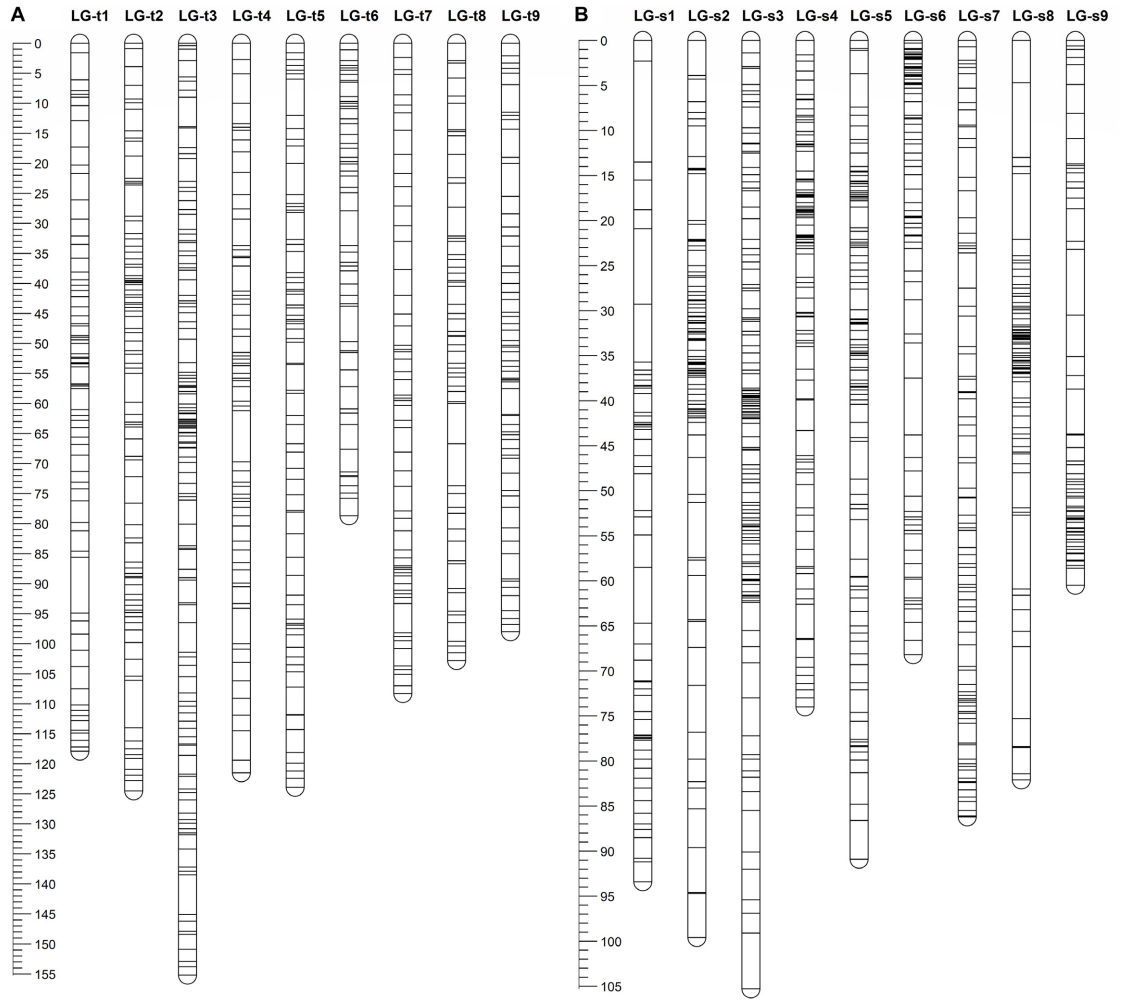


**FIGURE 1 |** Distribution of markers with unique loci in the genetic linkage map of trifoliate orange **(A)** and sweet orange **(B)**. The nine linkage groups correspond to the nine major scaffolds of the Clementine mandarin genome. Map distances in centiMorgans (cM) are indicated by the ruler at the left.

list remained stable. Kruskal–Wallis (KW) test was also used to provide complementary validation for significant genotypic means. Each significant QTL was characterized by its LOD score, percentage of explained phenotypic variation, confidence interval (in cM) corresponding to threshold LOD score and extension region at either side of the likelihood peak until the LOD score dropped to 2.0. QTLs that showed clearly overlapping confidence intervals were considered as co-localized. The position of QTLs on the genetic map was drawn using the MapChart 2.2 program (Voorrips, 2002).

# RESULTS

## RAD Sequencing and SNP-Based Genotyping

Four parental varieties ('Argentina' and 'Flying Dragon' trifoliate orange; 'Sanford' and 'Succari' sweet orange) and 170 $F_1$ progenies were processed for RAD sequencing on the NGS Illumina platform. Except one progeny, all had acceptable quality of sequencing reads. The average number of reads was 8.14E6 and 7.37E6 for trifoliate orange and sweet orange, respectively. The read counts for the 169 $F_1$ progenies ranged from 1.05E6 to 14.91E6, with an average of 4.53E6 per progeny. After alignment with the reference genome, the average number of read clusters was 1.83E5 and 1.47E5 for trifoliate oranges and sweet oranges, respectively, and the numbers of read clusters for the $F_1$ progenies ranged from 0.32E5 to 2.79E5 and averaged to be 1.03E5 (the detailed data are available in **Supplementary File S1**).

In total, 51,687 putative SNP loci were determined, of which 55.6% were transitions and 44.4% were transversions. Excluding SNP loci without calls, 96.7% of the genotyped SNP loci were identical between 'Argentina' and 'Flying Dragon' trifoliate orange, and 98.2% were identical between 'Sanford' and 'Succari' sweet orange (the detailed data are available in **Supplementary File S2**). Considering the low levels of genetic diversity found between the trifoliate orange parents and between the sweet orange parents, all the $F_1$ progenies from different crosses were

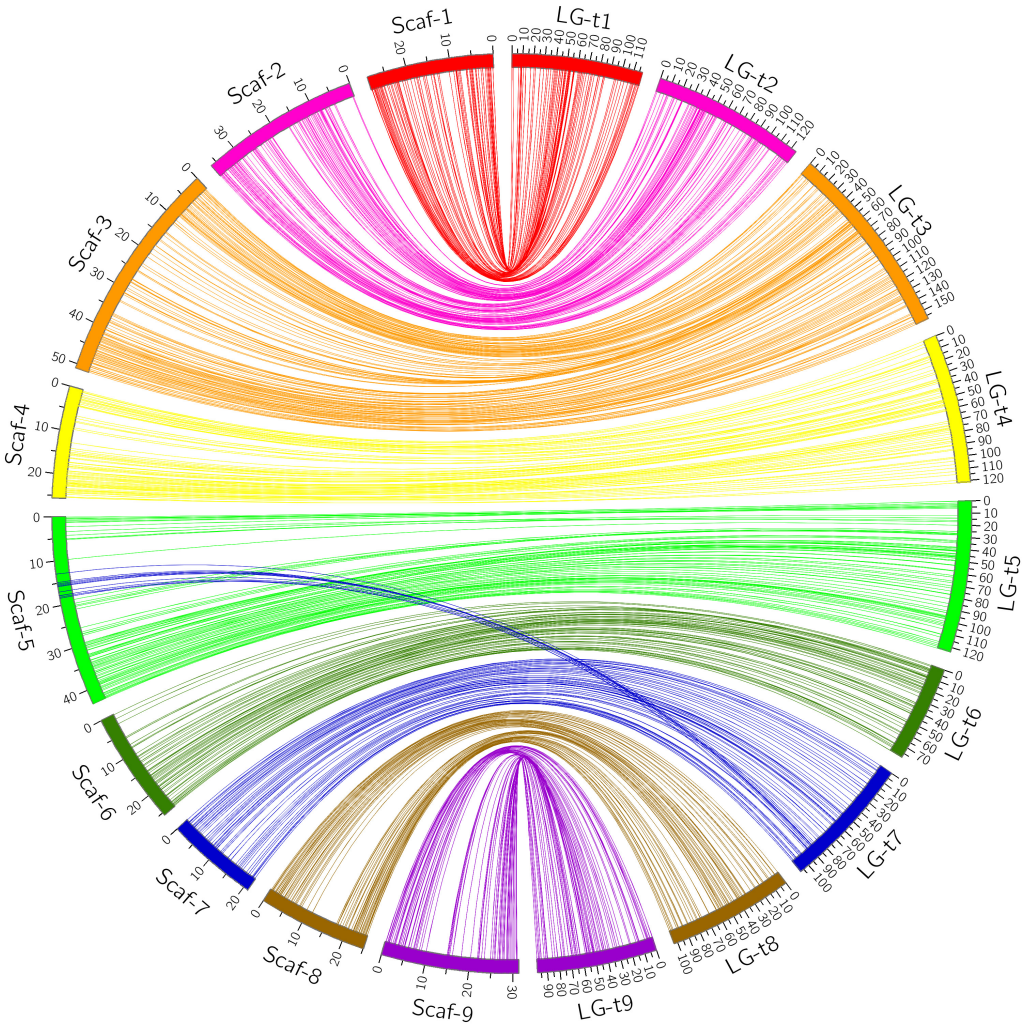


**FIGURE 2 |** Conservation of synteny and linear order of markers between trifoliate orange genetic map and Clementine mandarin genome via circle diagram.

combined as a single family. After stringent filtering, a total of 3,861 high quality SNP markers with configuration code "lm × ll" or "nn × np" were retained for genetic map construction, of which 1,408 SNP markers were heterozygous in trifoliate orange and 2,453 SNP markers were heterozygous in sweet orange.

## Genetic Linkage Map Construction and Evaluation

After eliminating markers with identical or similar segregation patterns that accounted for 62% of the filtered markers, all the remaining SNP markers in each dataset were grouped under the threshold LOD score of 4.0 into nine linkage groups, which is consistent with the haploid number of chromosomes in citrus. Additionally, all nine linkage groups conserved their integrity up to LOD of 10 for both parents. Finally, for trifoliate orange, a total of 647 high-quality SNP markers were mapped on nine linkage groups with unique loci, spanning a total genetic length of 1030.8 cM, with an average inter-locus distance of 1.59 cM (**Table 1** and **Figure 1A**). The number of markers within each linkage group ranged from 52 (for LG-t6) to 125 (for LG-t3), spanning a genetic distance ranging from 78.7 (for LG-t6) to 155.2 cM (for LG-t3). In total, 85.5% of the inter-locus gaps on the genome-wide genetic map were smaller than 3 cM and no gap was larger than 10 cM. For sweet orange, 754 high-quality SNP markers with unique loci were mapped into nine linkage groups, spanning a total genetic length of 760.2 cM with an average inter-locus distance of 1.01 cM (**Table 1** and **Figure 1B**). The number of markers within each linkage group ranged from 57 (for LG-s1) to 118 (for LG-s3), spanning a genetic distance ranging from 60.5 (for LG-s9) to 105.3 cM (for LG-s3). Of the inter-locus gaps on the whole genetic map, 93.3% were smaller than 3 cM and only one gap was larger than 10 cM (11.2 cM on LG-s1).

Through alignment, all SNP markers on the two genetic maps were successfully mapped onto nine major scaffolds of the Clementine mandarin genome. For trifoliate orange, except for 10 markers of LG-t7 which mapped on Scaffold_5, all other

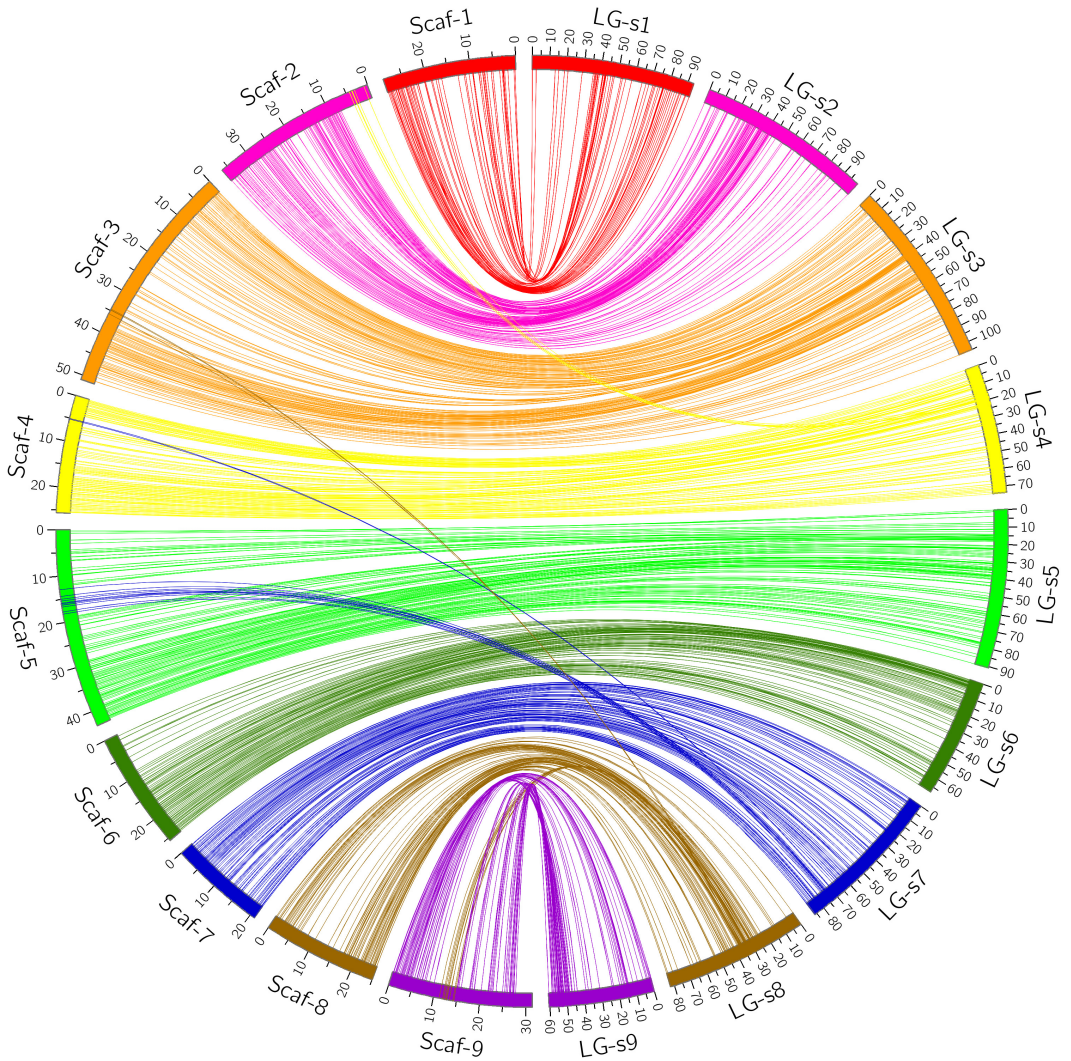


**FIGURE 3 |** Conservation of synteny and linear order of markers between sweet orange genetic map and Clementine mandarin genome via circle diagram.

637 markers were mapped onto syntenic scaffolds (**Table 1** and **Figure 2**). The overall coverage ratio of mapped markers on the Clementine genome was 98.6%, and the coverage ratio on each scaffold ranged from 95.5 (for LG-t7) to 99.7% (for LG-t5). For sweet orange, except for a total of 30 markers on LG-s4, LG-s7 and LG-s8, all other 724 markers were mapped onto syntenic scaffolds (**Table 1** and **Figure 3**). The overall coverage ratio of mapped markers on the Clementine genome was 95.0%, and the coverage ratio on each scaffold ranged from 87.7 (for LG-s9) to 99.3% (for LG-s8). Only some minor discrepancies were observed between the genetic maps and the Clementine genome, possibly due to genetic divergence among different citrus species, potential errors in the current linkage grouping, or erroneous assemblies in the reference genome. Collinear analysis of the consensus between the genetic map and the Clementine genome via a dot-plot diagram not only showed variations of the ratios between genetic distance to physical distance, but also clearly revealed high consensus between the genetic maps of trifoliate orange and sweet orange (**Supplementary Figure S1**).

## Phenotyping of HLB Infection

The phenotyping population consisted of 86 $F_1$ progenies randomly chosen from the above-mentioned mapping population, as well as six trifoliate oranges, two sweet oranges, and the rootstock seedings, with eight clonal replicate trees for each of individuals. After exposure to intense HLB pressure for 3 years in a replicated field trial, all phenotyped trees were diagnosed for *C*Las infection by the TaqMan label-based multiplex real-time PCR during the period of disease evaluation. As shown in **Table 2**, relatively few trees of trifoliate orange varieties were determined to be HLB-positive; in contrast, most trees of sweet orange varieties were HLB-positive. Meanwhile, $F_1$ progenies were mostly infected by *C*Las in both 2015 and 2016. For the HLB-negative genotypes, it should be noted that these trees were not confirmed to be immune to *C*Las, but

**TABLE 2** | Diagnosis of *C*Las infection and evaluation of severity level of HLB disease among control varieties and $F_1$ progenies.

| | | Hamlin | Navel | Argentina | Flying-dragon | Large-flower | Pomeroy | Rich 16-6 | Rubidoux | Volkamer | Progenies |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Sweet orange | Sweet orange | Trifoliate orange | Trifoliate orange | Trifoliate orange | Trifoliate orange | Trifoliate orange | Trifoliate orange | Rootstock | Hybrids |
| CLas | 2015 | 83.3% | 89.3% | 25.0% | 8.3% | 15.6% | 22.9% | 8.3% | 25.0% | 78.1% | 79.8% |
| infection[a] | 2016 | 94.4% | 100.0% | 15.0% | 7.6% | 27.9% | 33.5% | 12.9% | 25.0% | 80.0% | 87.4% |
| Foliar | 2015 | 4.1 ± 0.1 | 3.9 ± 0.1 | 1.1 ± 0.1 | 1.7 ± 0.3 | 1.6 ± 0.2 | 1.2 ± 0.1 | 1.4 ± 0.1 | 1.3 ± 0.1 | 2.9 ± 0.1 | 3.3 ± 0.1 |
| symptom[b] | 2016 | 3.8 ± 0.3 | 3.6 ± 0.2 | 1.4 ± 0.2 | 1.9 ± 0.1 | 1.4 ± 0.1 | 1.4 ± 0.1 | 1.2 ± 0.1 | 1.4 ± 0.1 | 2.3 ± 0.1 | 3.0 ± 0.1 |
| Canopy | 2015 | 3.8 ± 0.3 | 4.5 ± 0.3 | 1.3 ± 0.2 | 2.0 ± 0.3 | 1.3 ± 0.1 | 1.3 ± 0.2 | 1.4 ± 0.1 | 1.5 ± 0.2 | 1.2 ± 0.1 | 2.7 ± 0.1 |
| damage[b] | 2016 | 3.7 ± 0.3 | 4.4 ± 0.3 | 1.1 ± 0.1 | 2.0 ± 0.3 | 1.3 ± 0.1 | 1.0 ± 0.1 | 1.1 ± 0.1 | 1.1 ± 0.1 | 1.1 ± 0.1 | 2.6 ± 0.1 |

[a]*The status of CLas infection for all control varieties, indicated with the percentage of HLB-positive trees diagnosed by the TaqMan label-based real-time qPCR, were based on results of replicate trees; but for $F_1$ progenies, were the averaged results of 86 individuals.* [b]*The rating scores and variations for all control varieties, shown as Mean ± SE (n = 8), were based on scores of replicate trees; but for $F_1$ progenies, were the averaged means and variations of 86 individuals.*

they mostly had low titers of *C*Las (C*t* value of *C*Las diagnosis between 33 and 39), which might indicate partial resistance to *C*Las infection. The results indicate that all trees of the phenotyping population were adequately inoculated with *C*Las under continuous reinfection (the detailed data are available in **Supplementary File S3**).

The evaluation of HLB disease among the phenotyping population was conducted in 2015 and 2016 through repeated rating of the foliar symptom and canopy damage separately (**Table 2**). The ratings of foliar symptom in trifoliate oranges (ranged from 1.1 to 1.9) were significantly lower than those in sweet oranges (ranged from 3.6 to 4.1). For $F_1$ progenies, the ratings of foliar symptom ranged from 1.3 to 4.7, and the average rating was 3.3 in 2015 and 3.0 in 2016. The ratings of canopy damage in trifoliate oranges (ranged from 1.0 to 2.0) were also significantly lower than those in sweet oranges (ranged from 3.7 to 4.5). For $F_1$ progenies, the ratings of canopy damage ranged from 0.9 to 4.1, and the average rating was 2.7 in 2015 and 2.6 in 2016. The frequency distributions of foliar symptom and canopy damage ratings among the $F_1$ progenies are illustrated in **Figure 4**. As shown, obvious quantitative variation of the two traits were observed in both years. The correlation coefficient of phenotypic data between 2 years was 0.77 for foliar symptom rating and 0.91 for canopy damage rating. The correlation coefficients between two traits ranged from 0.47 to 0.74 and 0.58 in average, reflecting low consistency between the traits of disease in the progenies (the detailed data are available in **Supplementary File S4**).

## Detection of QTLs Associated With HLB

QTLs associated with the two phenotypic traits were detected separately in two parental genetic maps for each year (**Table 3**). In trifoliate orange, six and three QTLs associated with foliar symptom, and three and two QTLs associated with canopy damage, were identified in 2015 and 2016, respectively. The maximum LOD score for each of the QTLs ranged from 2.7 to 5.5, explaining an estimated phenotypic variation ($R^2$) ranging from 13.9 to 29.9%. In sweet orange, two QTLs associated with foliar symptom were identified in both 2015 and 2016, but no significant QTL associated with canopy damage was identified in either of the 2 years. The maximum LOD score for each of the QTLs ranged from 3.1 to 5.5, explaining an estimated phenotypic variation ($R^2$) ranging from 17.3 to 29.1%. None of the QTLs alone could explain a majority of the phenotypic variation, but they collectively explained a major part. The graphics of QTL mapping are available in **Supplementary Figure S2**.

Based on the confidence intervals of the QTLs on the genetic maps, most of the QTLs are repeatable between the 2 years (**Figures 5**, **6**). For the QTLs associated with foliar symptoms, seven QTLs were co-localized in three locations of the trifoliate orange map, on LG-t6, LG-t8 and LG-t9, while four QTLs were co-localized in two locations of LG-s7 on the sweet orange map. For the QTLs associated with canopy damage, only two QTLs on LG-t6 showed certain co-localization. It is noteworthy that co-localization of QTLs between the two traits were also observed in three locations of the trifoliate orange map, respectively, on LG-t6, LG-t8 and LG-t9. Overall, all the locations of QTLs

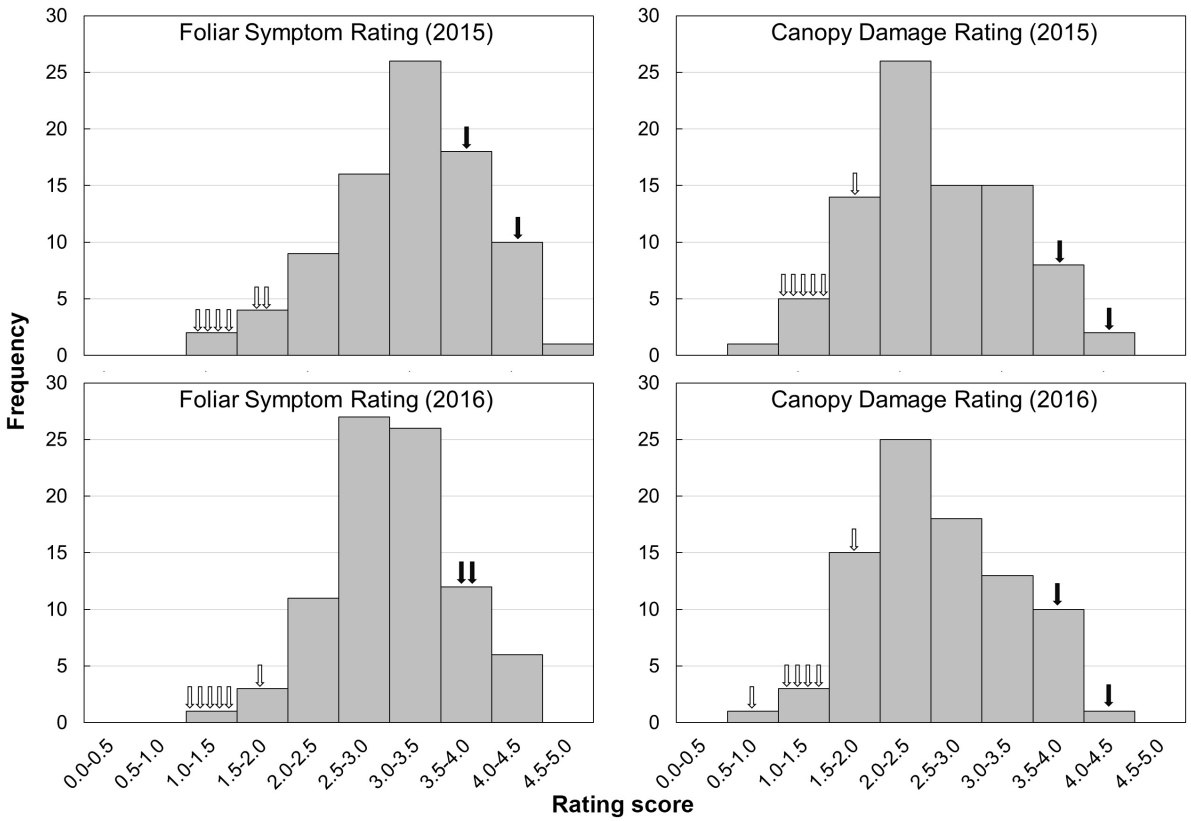


**FIGURE 4 |** Frequency distributions of foliar symptoms rating and canopy damage rating in $F_1$ progenies and parental varieties in 2 years. The hollow arrows indicate each of the trifoliate orange varieties, while the solid arrows indicate each of the sweet orange varieties.

associated with the two traits could be grouped into four main clusters, respectively, located on LG-t6, LG-t8, LG-t9 and LG-s7. The approximate genomic size of corresponding regions on the reference genome for these QTL clusters were Scaffold_6, Scaffold_8, Scaffold_9 and Scaffold_7. Among the QTL clusters, the one on LG-t6 was the most repeatable QTL and explained the largest part of the phenotypic variation.

# DISCUSSION

## Genotyping and Linkage Mapping

In this study, we constructed two separate parental genetic maps, which, to our knowledge, are the highest density genetic maps to date for trifoliate orange and sweet orange. The former highest density genetic map of trifoliate orange consisted of 146 SNP markers and 74 SSR markers, spanning a total genetic length of 937.1 cM and having an average density of 0.23 marker/cM (Lyon, 2008). Our trifoliate orange map consisted of 647 unique-loci SNP markers with an average density of 0.63 marker/cM and had quite high coverage of the citrus genome. Thus, our genetic mapping effort has greatly improved the saturation of the genetic map of trifoliate orange. This new linkage map can be utilized as a reference map in trifoliate orange genome assembly in the future. For sweet orange, the previously reported highest density genetic map consisted of 799 SNP markers and 189 SSR markers spanning a total genetic length of 1026.6 cM with an average density of 0.95 marker/cM (Lyon, 2008). The map was used as a reference map for the assembly of a sweet orange genome (Xu et al., 2013). We achieved a slightly higher density in our genetic map (0.99 marker/cM), though the map consisted of less markers. It is probably attributed to better quality of the genotyping and linkage mapping, resulting in smaller size of the linkage groups for our sweet orange map.

However, citrus genetic maps require substantial additional effort to achieve the high resolution found in model species genetic maps, which include thousands of markers with high accuracy and precision. Although we initially developed thousands of SNP markers for our linkage mapping, only a small portion of these markers were successfully mapped as unique loci. This has been observed in all previous SNP-based genetic maps of citrus, where many markers were located in "zero recombination clusters", indicating nearly no meiotic recombination occurs between markers within those regions (Lyon, 2008; Shimada et al., 2014; Guo et al., 2015; Yu et al., 2016; Curtolo et al., 2017; Imai et al., 2017). As shown in **Figures 3** and **4**, many markers in some small regions (<2 cM) of the linkage groups fell into clusters, which all correspond to very large genomic regions (5–10 Mb) on citrus chromosomes. High redundancy of markers is commonly attributed to small

**TABLE 3 |** QTLs detected separately in trifoliate orange and sweet orange genetic maps.

| Trait | QTL name | Time | LG[a] | Thold LOD[b] | Max LOD[c] | QTL position[d] | $R^2$ (%)[e] | Nearest marker[f] | Marker position[g] | Allele type[h] | KW significance[i] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Foliar symptom | FS-2015-t6a | 2015 | t6 | 2.7 | 4.0 | 43.8 | 20.9 | 6_19748604 | 43.8 | A/G | ****** |
| | FS-2015-t6b | 2015 | t6 | 2.7 | 3.9 | 55.4 | 20.8 | 6_21328107 | 54.4 | T/C | ******* |
| | FS-2015-t8a | 2015 | t8 | 2.7 | 4.3 | 39.5 | 22.1 | 8_5646672 | 39.5 | G/A | ****** |
| | FS-2015-t8b | 2015 | t8 | 2.7 | 5.0 | 54.9 | 24.6 | 8_18164251 | 54.9 | T/C | ******* |
| | FS-2015-t9a | 2015 | t9 | 2.7 | 4.8 | 66.0 | 24.5 | 9_25649934 | 66.0 | A/T | ******* |
| | FS-2015-t9b | 2015 | t9 | 2.7 | 3.8 | 83.9 | 20.8 | 9_28915131 | 82.9 | A/G | ******* |
| | FS-2016-t6 | 2016 | t6 | 2.7 | 5.5 | 47.8 | 29.9 | 6_20249564 | 49.7 | A/G | ******* |
| | FS-2016-t8 | 2016 | t8 | 2.7 | 2.7 | 55.6 | 13.9 | 8_18728386 | 55.6 | T/C | **** |
| | FS-2016-t9 | 2016 | t9 | 2.7 | 3.2 | 83.9 | 18.1 | 9_29102006 | 84.9 | C/T | ****** |
| | FS-2015-s7a | 2015 | s7 | 2.6 | 4.7 | 56.3 | 25.3 | 7_11398231 | 56.3 | A/T | ******* |
| | FS-2015-s7b | 2015 | s7 | 2.6 | 5.5 | 67.1 | 29.1 | 7_15491756 | 67.1 | T/C | ******* |
| | FS-2016-s7a | 2016 | s7 | 2.6 | 3.5 | 43.9 | 19.5 | 7_7947819 | 43.9 | C/T | ****** |
| | FS-2016-s7b | 2016 | s7 | 2.6 | 3.1 | 73.5 | 17.3 | 8_20039431 | 73.5 | G/A | ****** |
| Canopy damage | CD-2015-t6 | 2015 | t6 | 2.7 | 3.7 | 43.4 | 22.0 | 6_19837597 | 43.4 | T/C | ******* |
| | CD-2015-t8 | 2015 | t8 | 2.7 | 3.0 | 50.2 | 16.8 | 8_8274031 | 50.2 | C/G | **** |
| | CD-2015-t9 | 2015 | t9 | 2.7 | 3.5 | 59.5 | 21.2 | 9_22115818 | 57.5 | A/T | ****** |
| | CD-2016-t6 | 2016 | t6 | 2.7 | 2.8 | 27.9 | 14.6 | 6_17324675 | 27.9 | C/A | **** |
| | CD-2016-t7 | 2016 | t7 | 2.7 | 3.0 | 35.0 | 15.9 | 7_4403223 | 33.0 | C/A | ***** |

*[a]The linkage group of trifoliate orange (t1–t9) or sweet orange (s1–s9) where QTL was detected. [b]The threshold LOD score was determined by 1,000 permutation tests in the genome-wide map. [c]The maximum LOD score of each QTL was determined by Interval Mapping. [d]The QTL position (in cM) from the top of LG corresponding to the maximum LOD score. [e]The percentage of the total phenotypic variation explained by QTL. [f]The name of the nearest marker also represent the genomic position in the reference genome. [g]The position (in cM) of the nearest marker from the top of the corresponding linkage group. [h]The allele nucleobase type of the nearest marker with the first base unique in the detected parent. [i]The significance level of the Kruskal–Wallis test: ****$p < 0.0001$; *****$p < 0.00001$; ******$p < 0.000001$; *******$p < 0.0000001$.*

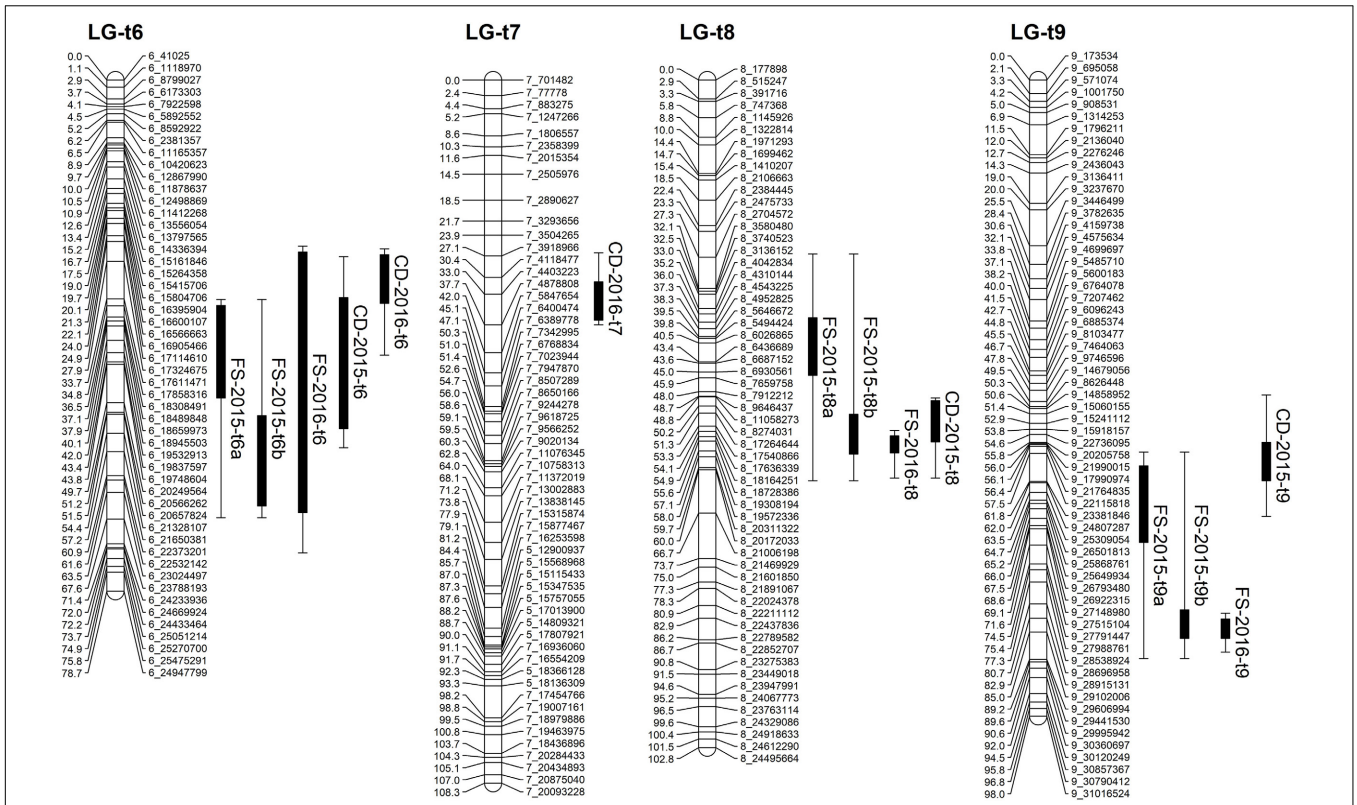


**FIGURE 5 |** Mapping QTLs with foliar symptoms and canopy damage on the trifoliate orange genetic linkage map. Thick bars on the right side of each LG indicate confidence interval of QTLs with LOD score above threshold and flanking error bars indicate extension of QTL region at LOD score 2.0.

population size, closely adjacent physical marker location, and regions with low recombination, which could be significantly improved by increasing the size of mapping populations and the evenness of marker distribution. These solutions have been applied to mapping of many plant species but are difficult for citrus species. Biological characteristics of citrus, such as long juvenility, seedlessness, polyembryony, apomixis, heterozygosis, gametophytic incompatibility, zygotic selection and gametic selection (Shimada et al., 2014), not only seriously hamper the development of numerous uniquely segregating markers and production of large full-sib populations, but also remarkably influence allelic segregation and recombination ratio (Bernet et al., 2010; Ollitrault et al., 2012a). In citrus hybridizations, parental genotype and homology have obvious effects on genetic map density. Guo et al. (2015) successfully constructed an integrated genetic map of pummelo with 1543 SNP markers using an intraspecific full-sib $F_1$ population with only 124 individuals. By contrast, Curtolo et al. (2017) attempted to construct a genetic map using an interspecific full-sib $F_1$ population with 278 individuals from crossing of tangor and sweet orange, but only 661 non-redundant SNP-based DArTseq markers were finally mapped on the integrated map. The differences indicate that the use of intraspecific populations tends to achieve much higher marker density in the construction of genetic maps than interspecific populations, even though the population size is much smaller. In our study, the mapping population is neither interspecific nor intraspecific hybrids, but intergeneric hybrids, which had made it more difficult to achieve high-density genetic maps. The potential karyotypic and genomic divergences between trifoliate orange and sweet orange can prevent normal meiotic pairing and homology recombination during meiosis. Some genomic divergences between the two species can be obviously observed from the conservation of synteny of the genetic linkage maps and the reference genome, e.g., the divergences on LG-4, LG-7 and LG-8. In addition to parental genotype, differential fitness of gamete genotypes, crossing direction, and regulatory gene interactions likely also contribute to the high level of segregation distortion in citrus (Bernet et al., 2010; Curtolo et al., 2017). It should be noted that our mapping population was not a full-sib family, but mixed progenies from three different crosses between two trifoliate orange varieties and two sweet orange varieties. Our genotyping results show that only 3.4 and 1.8% of genotyped SNP loci are inconsistent, respectively, between the trifoliate oranges and sweet oranges. However, when compared to the identified segregating SNP markers, the percentages rise up to 29.6 and 6.1%, respectively (**Supplementary File S2**). This is a substantial percentage of inconsistent segregating markers in trifoliate orange. By analyzing the distribution of these inconsistent SNP loci in trifoliate orange on the reference genome, we found that they are mostly concentrated in certain regions of the genome, and the two trifoliate oranges almost share all of these

regions. Therefore, elimination of these polymorphic loci not only reduced the number of available segregating SNP markers, especially for the trifoliate orange map, but also resulted in several large regions with fewer markers on the genetic maps. Actually, many large gaps on the genetic maps were rightly located in the positions of the eliminated polymorphic SNP loci. In addition, in comparison to sweet orange, many fewer segregating markers were identified in trifoliate orange (**Supplementary File S2**). This problem was reported in all previous genetic maps of trifoliate orange (Chen et al., 2008; Lyon, 2008; Ollitrault et al., 2012a). The low availability of segregating markers in trifoliate orange is due to its lower heterozygosity. As the current sequence coverage only accounts for 2.3% of the citrus genome, to maximize detection of segregating SNP markers, greater sequence coverage is needed for the progenies and parents.

Significant differences in linkage group sizes were observed between trifoliate orange and sweet orange genetic maps. Each of the linkage groups of trifoliate orange is larger in genetic length than the corresponding one of sweet orange, even though there were less markers mapped on the trifoliate orange genetic map. Similar differences in genetic distances were evident in the previously reported EST-SSR genetic maps based on codominant markers segregating in both parents (Chen et al., 2008). Variation in map length could also be observed between SNP-based genetic maps of mandarin, pummelo and sweet orange (Ollitrault et al., 2012a). Due to the utilization of a reference genome, SNP-based genetic linkage maps are comparable if referred to physical distance. The variation in genetic map length is unrelated to genome size among different citrus species, which range from 360 to 398 Mb (Gmitter et al., 2012; Wu et al., 2014, 2018). Extensively distributed in our maps, nearly half of the markers exhibited significant segregation distortion, and such segregation distortion in citrus was proposed to result from gametic selection rather than zygotic selection (Ollitrault et al., 2012a). However, in a simulation study on factors affecting linkage map construction, segregation distortion from gametic selection had little influence on marker order and genetic distance (Hackett and Broadfoot, 2003). Thus, variation of genetic size between linkage group maps of trifoliate orange and sweet orange probably is not due to segregation distortion, but reflects differential recombination rates between the species. Studies on model plants amply demonstrate the impact of genome sequence divergence on recombination rate, and lower recombination rate is related to higher levels of genome divergence (Chetelat et al., 2000; Opperman et al., 2004; Li L. et al., 2006). For different citrus-related genera and species, the degree of genome heterozygosity differs dramatically. Sweet orange, known to be highly heterozygous, was demonstrated to be a complex interspecific hybrid derived from pummelo and mandarin (Xu et al., 2013; Wu et al., 2014). However, trifoliate orange, a genus related to *Citrus*, was found to be lower in heterozygosity (Chen and Gmitter, 2013). Therefore, in comparison to trifoliate orange, higher heterozygosity in the sweet orange genome probably suppresses recombination frequency, resulting in a smaller genetic size. This is in agreement with the difference in genetic distance between shared markers on genetic maps of Clementine mandarin and pummelo (Ollitrault et al., 2012a).

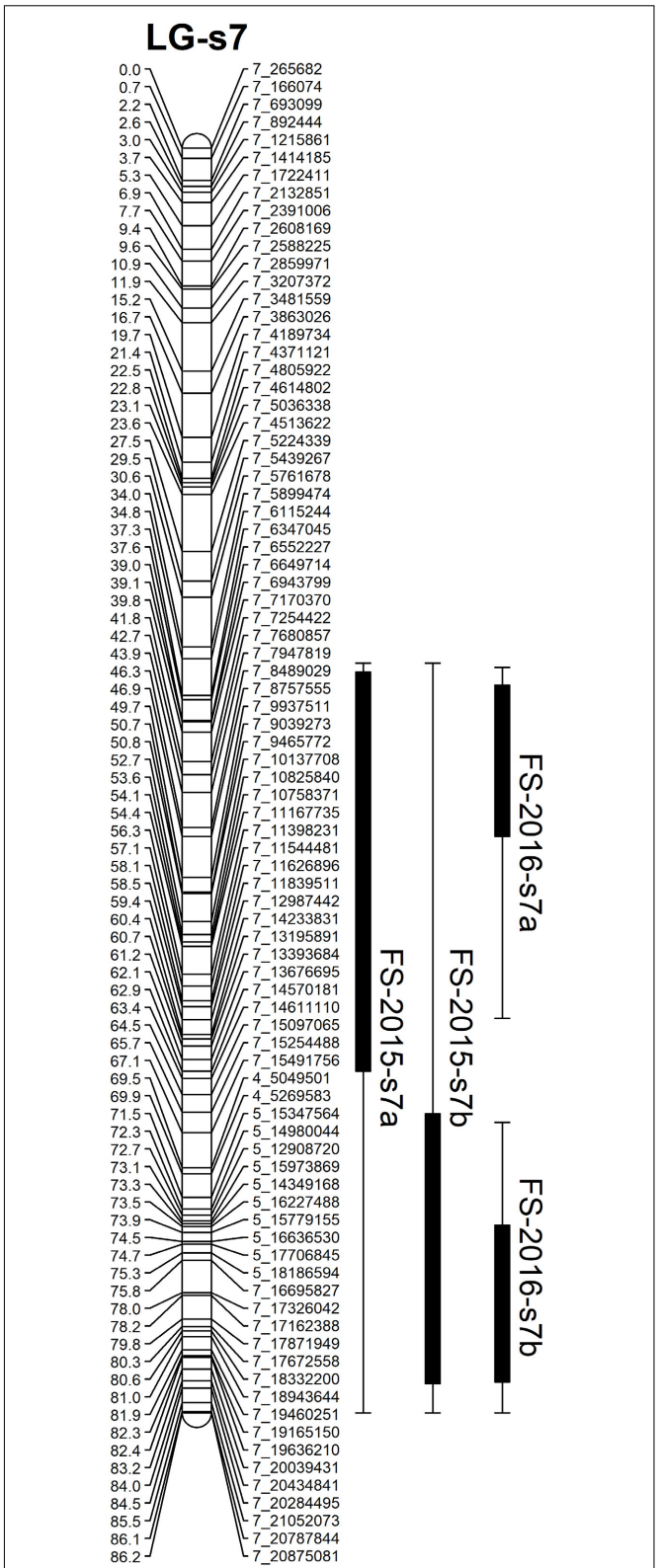


**FIGURE 6 |** Mapping QTLs with foliar symptoms and canopy damage on the sweet orange genetic linkage map. Thick bars on the right side of each LG indicate confidence interval of QTLs with LOD score above threshold and flanking error bars indicate extension of QTL region at LOD score 2.0.

Clementine mandarin is an interspecific hybrid of *C. reticulata* × *C. sinensis* with high genome heterozygosity (Wu et al., 2014), while pummelo is a progenitor species in *Citrus* with low genome heterozygosity (Wang X. et al., 2017). In addition, recombination rates are known to differ between sexes in both plants and animals (Lorch, 2005). The size of the male genetic map of Clementine mandarin was notably larger than its female genetic map (Ollitrault et al., 2012a). Our mapping population was a mix of several crosses between different varieties of trifoliate orange and sweet orange with taxa serving as male and female parents in different crosses. However, most of the progenies were generated with trifoliate orange as the male parent, which may also contribute to the larger size of the trifoliate orange genetic map.

## Phenotyping and QTL Mapping

This is the first report on identification of QTLs related to HLB disease and tolerance. Most of the recently reported QTLs in citrus are related to morphological and physiological traits (Kepiro and Roose, 2010; Sugiyama et al., 2011; Sahin-Cevik and Moore, 2012; Raga et al., 2014; Asins et al., 2015; Raga et al., 2016; Yu et al., 2016; Curtolo et al., 2017; Imai et al., 2017; Yu et al., 2017). Only a few reports focused on disease-related QTLs in citrus, such as resistance to citrus tristeza virus (CTV) (Asins et al., 2012; Ohta et al., 2015), Alternaria brown spot (ABS) (Cuenca et al., 2013, 2016), citrus leprosis virus (CiLV) (Bastianel et al., 2009), and citrus nematode (Ling et al., 2000). In genetic studies, estimated effects of each QTL for disease resistance in plants usually ranges from a few percent to 50% or more, and a QTL accounting for phenotypic variation of more than 20% is commonly described as a major QTL, or more than 50% as a dominant QTL (Davey et al., 2006; St Clair, 2010). Interestingly, only one QTL was found with a dominant effect on the phenotypic resistance for each of the diseases, suggesting that the inheritance of resistance for these diseases is mainly controlled by a single dominant allele. In our study, four clusters of QTLs associated with HLB tolerance were identified on the two parental genetic maps. Although all of them could be considered as major QTLs, none of them alone could explain a majority of the phenotypic variation. Our results indicate that the high degree of tolerance to HLB in trifoliate orange cannot be monogenic, because at least four genomic regions are involved. However, the HLB pathogenesis mechanism is still unknown and is likely complicated (Martinelli and Dandekar, 2017;Wang N.A. et al., 2017). Based on the most recent progress in studies on HLB, it was proposed that at least three main molecular mechanisms occur in citrus in response to HLB, and these mechanisms involve many pathways and genes (Martinelli and Dandekar, 2017). This is in agreement with our results that citrus tolerance to HLB is polygenic.

Unlike CTV, CiLV, ABS and citrus nematode for which strong resistance is available within the citrus gene pool, the suppression of HLB in trifoliate orange may be best described as tolerance. Trifoliate orange had been reported as resistant to *C*Las infection (Folimonova et al., 2009), but recently more studies suggested that it is not true resistant but highly tolerant (Ramadugu et al., 2016; Miles et al., 2017). In our study, though the trifoliate oranges were generally determined to be HLB-negative, most of the replicate trees had marginal results for *C*Las diagnosis (C*t* value of qPCR ranging from 33 to 39), and a few trees were even HLB-positive (C*t* value of qPCR under 33). It is important to note that at least eight replicate trees were clonally propagated for each genotype and they all were used throughout the phenotyping, which ensured high accuracy and reliability of the phenotypic results for each genotype. Thus, we believed that the trifoliate orange was probably infected by *C*Las, but the *C*Las titer was held at relatively low levels. Our results on HLB disease evaluation also support this interpretation. The trifoliate oranges were not completely healthy under the intense HLB pressure, but mostly displayed slight foliar disease symptoms and slight canopy damage. The results further suggest that trifoliate oranges are not immune to *C*Las infection, but can inhibit growth of *C*Las and show low symptom levels when they are infected. It is noteworthy that obviously different degrees of tolerance to HLB was observed among the $F_1$ progenies, and a few progenies showed similar results of *C*Las diagnosis and disease evaluation as trifoliate oranges, indicating the HLB tolerance is inheritable. Moreover, some other hybrids containing the germplasm of trifoliate orange were also found with certain tolerance to HLB (Albrecht and Bowman, 2011, 2012; Ramadugu et al., 2016), probably due to the partial inheritances of HLB tolerance from trifoliate orange.

Although high-density genetic maps were constructed for both trifoliate orange and sweet orange, the outcome of QTL mapping was still not fine enough for anchoring specific genomic regions or specifying candidate genes associated with citrus responses to HLB. The four anchored genomic regions spanned a total genomic length of approximately 44.6 Mb, consisting of thousands of genes. These genomic regions involved 57 markers from three regions of the trifoliate orange map and 54 markers from one region of the sweet orange map, indicating the marker density of our genetic maps is sufficient for fine mapping. One possible explanation for such phenomenon could be that the quality of the constructed genetic maps is still insufficient for fine mapping of QTLs. The characteristics of plant materials themselves and the utilization of mixed intergeneric populations for genotyping likewise may restrict the construction of genetic maps with sufficient resolution and accuracy. All the linkage groups in trifoliate orange where the QTLs were detected were constructed with fewer markers, especially within the regions of some QTLs. Another possible explanation could be that the size of the phenotyping population is not large enough to yield fine phenotypic data with wide variation. Due to the space requirements for trees and test costs, only 86 of the progenies were selected for phenotyping in our study, accounting for only a half of the genotyping population. Moreover, it is also possible that each of these regions consist of multiple QTLs and they generally co-segregated in the population. As presented in the graphs of QTLs, many QTLs have more than one peak within the LOD score profile (**Supplementary Figure S2**). Increasing the size of the phenotyping population, to equal that of the genotyping population, should greatly improve the quality of QTL mapping and minimize the number of anchored genomic

regions associated with HLB tolerance; however, such larger experiments with tree crops can become cost-prohibitive, in comparison with similar experimental designs with annual or model plant species.

## CONCLUSION

Based on Genotyping-by-Sequencing of an intergeneric $F_1$ population of 170 progenies, we constructed two high-density genome-wide genetic maps for trifoliate orange and sweet orange. Each of the genetic maps contained nine firm linkage groups corresponding to the haploid chromosome number of the species and exhibited high synteny and high coverage of the reported citrus genome. The minor discrepancies among the genetic maps and genome assemblies may represent possible structural rearrangements among citrus species, or alternatively errors in the previous genome assembly. In the replicated field evaluation over 2 years, trifoliate orange and sweet orange showed significant differences in response to *C*Las infection and HLB, and their progenies exhibited an obvious continuous distribution for two phenotypic traits. Four clusters of QTLs were identified for HLB-incited foliar and canopy responses, respectively, located on LG-t6, LG-t8 and LG-t9 of the trifoliate orange genetic map and LG-s7 of the sweet orange genetic map. These QTLs collectively explained a major part of the phenotypic variation in response to HLB disease. Our results suggest that multiple QTLs are involved in the genetic control of HLB response in citrus. This work provides a starting point for future studies of the underlying genetic architecture of resistance or tolerance to HLB. These QTLs need to be confirmed further to facilitate breeding for resistance or tolerance to HLB in citrus. In addition, the corresponding genomic regions need to be refined if the objective is to discover and characterize candidate genes related to the host response to disease. The final identified QTLs and genes could be good targets for citrus breeding to support long-term solutions to this devastating disease.

## AUTHOR CONTRIBUTIONS

FG, ES, MR, ZD, and MH conceived the study. FG and MR developed the mapping population. MR, MH, and QY conducted the work of genotyping. MH, QY, DD, YY, and YZ conducted the work of phenotyping. MH and MR analyzed the genotypic data. MH analyzed the phenotypic data, performed the QTL mapping, and drafted the manuscript. All authors read and approved the final manuscript.

## FUNDING

This work was partly supported by grants from the Citrus Research and Development Foundation Inc. (CRDF #536, #15-010) and the New Varieties Development and Management Corporation (NVDMC), on behalf of the Florida citrus industry, and from USDA-NIFA-SCRI #2015-70016-23027.

## ACKNOWLEDGMENTS

The authors thank Michael Irey for testing the samples; Randy Driggers for propagating and planting the trees and Sean Reif for maintenance of the field trees; Peng Ling, Yanbo Wang, Misty Holt, Fabieli Irizarry and Hui Wen for their assistance in sampling.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01694/full#supplementary-material

**FIGURE S1 |** Collinear analysis of consensus between genetic linkage maps and reference genome by dot-plot diagram.

**FIGURE S2 |** Full genetic linkage maps and genome-wide LOD score graphics of QTL mapping of two phenotypic traits.

**FILE S1 |** Summary of Genotyping by Sequencing for 170 $F_1$ progenies and four parental varieties.

**FILE S2 |** Analysis of parental genotypes and distribution of SNP loci on reference genome in four parental varieties.

**FILE S3 |** Diagnosis of *C*Las infection by real-time qPCR in 86 $F_1$ progenies and nine control varieties.

**FILE S4 |** Phenotypic data of two traits for the evaluation of HLB disease in 86 $F_1$ progenies and nine control varieties.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a shared affiliation, though no other collaboration, with several of the authors MH, QY, DD, YY, YZ, and FG.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 20 December 2018
doi: 10.3389/fpls.2018.01891

# Synthetic Ligands of Olfactory Binding Proteins Modulate Aggregation Response of Asian Citrus Psyllid in the Presence of Host-Plant Volatiles

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There is interest in using ligands of chemosensory binding proteins (CBP) to augment an insect's responsiveness to chemosensory cues. We showed previously that combining a synthetic ligand of a CBP with limonene, a common citrus volatile, enhanced the probing response of Asian citrus psyllid (*Diaphorina citri*). Here, we determined whether synthetic compounds, which were ligands of *D. citri* olfactory binding protein (OBP) DCSAP4, influenced the settling and aggregation levels of psyllids on young citrus shoots. The test ligands and Cmac scent were dispensed from a droplet of an emulsified wax product (SPLAT) placed on the bottom of each vial. The shoots were presented: (1) alone (shoot + blank SPLAT), (2) with a mixture of citrus volatiles ("Cmac scent") (shoot + SPLAT with Cmac scent), or (3) with different concentrations of test ligands (shoot + SPLAT with test ligand at concentration 1, shoot + SPLAT with test ligand at concentration 2, etc.). Depending on the availability of test ligands, sprigs, and psyllids, each test included from two to four replicates of each treatment (i.e., shoot only, shoot + Cmac scent, shoot + test ligand at concentration 1, shoot + test ligand at concentration 2, etc.); only a single test ligand was presented in each test. For each test, 200 *D. citri* were released in the test area and the numbers of psyllids on each sprig were counted 24 h later. Sprigs with ≥7 psyllids were considered to be an aggregation. A total of seven ligands were tested individually. Four of the ligands (654, 717, 784, and 861) modulated psyllid settling and aggregation response, causing greater settling and aggregation to sprigs presented with the Cmac scent than to those sprigs with blank SPLAT. Presentation of one of the ligands (019) resulted in an opposite effect in which psyllid settling and aggregation levels were lower on sprigs with Cmac scent than on those with blank SPLAT. There were no differences in settling levels in the different treatment vials in the Ligand 905 experiment. In the Ligand 937 experiment, settling levels did not vary significantly between treatment vials although settling levels were relatively high in all treatment vials and there was a significant treatment effect. Increased settling and aggregation levels were largely not observed with in the vials with only the test ligands, and there was little effect of ligand concentration on psyllid response levels.

OPEN ACCESS

**Edited by:**
*Alberto Urbaneja, Instituto Valenciano de Investigaciones Agrarias, Spain*

**Reviewed by:**
*Irina T. Sinakevitch, Arizona State University, United States*
*Sandra Vacas, Universitat Politècnica de València, Spain*

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**Specialty section:**
*This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Plant Science*

**Received:** *17 September 2018*
**Accepted:** *06 December 2018*
**Published:** *20 December 2018*

**Citation:**
*Patt JM, Meikle WG, Niedz RP and Woods D (2018) Synthetic Ligands of Olfactory Binding Proteins Modulate Aggregation Response of Asian Citrus Psyllid in the Presence of Host-Plant Volatiles. Front. Plant Sci. 9:1891. doi: 10.3389/fpls.2018.01891*

This suggests that the test ligands themselves did not attract the psyllids but rather modulated the psyllid's response to the Cmac scent. The results suggest that synthetic ligands of *D. citri* CBPs can be used to increase the effectiveness of citrus scent lures used to attract psyllids to monitoring traps and attract and kill devices.

**Keywords: citrus greening, insect attractant, *Diaphorina citri*, ligand, chemosensory binding protein, volatile, semiochemical**

## INTRODUCTION

The Asian citrus psyllid, *Diaphorina citri* (Kuwayama) (Hemiptera: Psyllidae) is the vector of *Candidatus* Liberibacter asiaticus, the causal agent of citrus greening disease or Huanglongbing (Halbert and Manjunath, 2004; Grafton-Cardwell et al., 2013; Hall et al., 2013). Citrus greening is the most devastating disease of citrus in the world today and has resulted in the loss of hundreds of thousands of hectares of citrus groves and billions of dollars in productivity (Bové, 2006; Gottwald, 2010; Wang and Trivedi, 2013; Spreen et al., 2014). ACP inhabits citrus trees in both commercial groves and residential areas and can move over large distances in a relatively short time (Boina et al., 2009; Tiwari et al., 2010; Martini et al., 2014; Lewis-Rosenblum et al., 2015).

Detection and monitoring efforts currently rely on tap sampling, direct visual counts, and yellow sticky card traps (Hall et al., 2007;Sétamou et al., 2008; Qureshi et al., 2009; Hall and Hentz, 2010; Hall et al., 2010); the former are labor intensive while the latter may provide inconsistent results. There is a great need for more reliable monitoring methods to effectively detect psyllid infiltration into citrus producing areas, monitor their population densities, and track the efficacy of control measures. *D. citri* is attracted to the young shoots of *Citrus* and closely related genera. The tender shoots are the only place where it mates, oviposits, and develops (Halbert and Manjunath, 2004; Grafton-Cardwell et al., 2013; Hall et al., 2013; Cifuentes-Arenas et al., 2018). The stimuli involved in host plant location and selection behavior by *D. citri* encompass a combination of visual cues (Wenninger et al., 2009b; Sétamou et al., 2014; Paris et al., 2015, 2017a,b), constitutive and induced host plant volatiles (Patt and Sétamou, 2010; Mann et al., 2012; Patt et al., 2014; Stockton et al., 2016; Patt et al., 2018), and conspecifics' pheromones (Wenninger et al., 2008; Martini et al., 2014; Stockton et al., 2017) and vibrational signals (Wenninger et al., 2009a; Rohde et al., 2013). Determining the identity of these stimuli is necessary to develop effective traps for detecting the presence of adult psyllids and monitoring their population density.

While visual cues are probably the primary long distance attractant for *D. citri*, olfactory cues could make an important contribution at close range to trap effectiveness (Patt and Sétamou, 2010). However, the complexity and variation inherent in citrus foliar volatiles makes it difficult to formulate scent lure mixtures with the composition, proportion, and concentration needed to function effectively.

There is potential for using ligands of olfactory binding proteins (OBPs) as surrogate scent attractants, repellents, or confusants, or to augment an insect's responsiveness to natural olfactory cues (Zhou et al., 2010). Although the *D. citri* antenna has relatively few sensillar hairs (Onagbola et al., 2008), transcriptome analysis revealed that these structures contain numerous olfactory receptors, OBPs, and associated proteins (Wu et al., 2016) and electrophysiological studies demonstrated response to a large number of volatile compounds from several chemical classes (Coutinho-Abreu et al., 2014a,b). To evaluate *D. citri* behavioral response to synthetic compounds identified as putative ligands of antennal OBPs, Patt et al. (2013) measured the number of salivary sheaths deposited in artificial midribs made from an emulsified wax, commonly called SPLAT (ISCA Tech, Inc.). They found that probing level was significantly higher in midribs containing a mixture of limonene and a particular synthetic ligand than in midribs containing either limonene or the ligand alone. This result indicated that the addition of an OBP ligand could enhance the responsiveness of *D. citri* to naturally occurring citrus foliar volatiles.

In this study, we used a settling vial assay to determine whether the presence of putative ligands of *D. citri* OBPs influenced the psyllid's settling and aggregation levels on young citrus shoots, both when the shoots were presented alone or in combination with a mixture of citrus volatiles.

## MATERIALS AND METHODS

### Study Plants and Insects

Psyllids used in the experiments were obtained from a colony of *D. citri* maintained at the USDA-ARS laboratory in Fort Pierce, FL, United States. Young sprigs were obtained from potted *Citrus macrophylla* (Wester) raised in a greenhouse at the same location.

### Selection of Novel Synthetic Ligands

The synthetic compounds were initially identified as novel ligands of OBPs using the Attenu assay, a proprietary, high throughput assay developed by Inscent, Inc. Attenu uses fluorescence quenching to identify the level of interaction between OBPs and potential ligands. When a putative ligand displaces a fluorescent marker from the binding pocket of an OBP, the reduction in fluorescence indicates that the test ligand reacted with the OBP. OBPs isolated from *D. citri* were used as screening targets to identify potential synthetic ligands. The most reactive compounds were then screened to measure influence on psyllid probing behavior (Patt et al., 2013). One synthetic compound, identification number 5276937, elicited the highest level of probing response by the psyllids. In the Attenu assay, this synthetic ligand reacted with OBP DcSAP4. All of the ligands tested in the present study were structurally related to Ligand

5276937. Because the ligands are proprietary, their structures are not shown here.

The test ligands used in the present study each had an identifying serial number, which was abbreviated to the last three digits for recording the results. They were identified as follows: 5267019 (="019"), 64288654 (="654"), 9325717 (="717"), 5649784 (="784"), 5655861 (="861"), and 6428905 (="905"). Ligand 5276937 (="937") was included in the present study because the other test ligands are structurally related to it and it was previously shown to increase behavioral responsiveness of *D. citri* (Patt unpublished data).

## Behavioral Assay

A modification of the settling assay used in Hall et al. (2015) and Patt et al. (2018) was used to evaluate psyllid settling and aggregation responses on citrus sprigs in the presence of either test ligands or synthetic host plant volatiles. The assay consisted of presenting the psyllids with a series of settling vials, each containing a citrus sprig and a scent dispenser consisting of a droplet of an emulsified wax product (SPLAT, ISCA Tech, Inc.) (Patt et al., 2011). The SPLAT droplet was placed in the center of a filter paper disk inserted into the bottom of the test vial and contained an aliquot of either a test ligand or an artificial citrus scent mixture. A blank SPLAT droplet was used in the negative control treatment.

A young sprig, ca. 2.5 cm long, was placed in an individual vial (25 dram clear-plastic vials, 39 mm diameter × 85 mm high (BioQuip Products, Inc., Gardenia, CA, United States) and capped with a white snap-on plastic lid. The sprigs were excised from potted *C. macrophylla* because the psyllids were reared on this plant and *D. citri* responds behaviorally more strongly to its natal host plant than to novel host plants (Patt et al., 2014; Stockton et al., 2016). The cut ends of the sprig was placed into a 1.3 mL Eppendorf vial filled with tap water; the Eppendorf tube was held in place with plastic holders glued to the inside of the vial. The vial cap had a series of 6 mm diameter holes to permit psyllid entry into the vial (**Figure 1**). Once inside the vial, psyllids strongly tended to feed and remain on the sprigs. The test ligands were dispensed *via* a 100 μL droplet of SPLAT applied to the center of a filter paper disk placed on the bottom of the test vial (**Figure 1**). White-colored SPLAT was used to preclude any confounding effects from visual attraction to the test odor source.

The vials were placed in an incubator fitted with fine screening to confine the psyllids in an area ca. 20.5 cm high × 67.0 cm wide × 41.5 long. The vials were arranged in two rows directly underneath fluorescent lights positioned 13.5 cm above the vial caps. The positions of the vials were randomized to prevent spatial bias. The lights were programed for a 16-h light: 8-h dark cycle. Two hundred adult psyllids, 5–10 days old (post-eclosion), were released into each incubator at the start of the test. After 24 h, the vials were sealed and placed in a freezer for 2 h. After freezing, the numbers of psyllids in each vial were counted.

Limited amounts of ligands were available for testing. When 100 mg of test ligand was available, the following concentrations were tested: 3-, 9-, 30-, and 90 mg OBP ligand/10 mL SPLAT. This concentration series was used for ligands 019, 654, and 937 (**Table 1**). When 50 mg of test ligand was available, concentrations of 9- and 30 mg OBP ligand/10 mL SPLAT were tested. This concentration series was used for ligands 717, 784, 861, and 905 (**Table 1**).

**FIGURE 1 |** Settling vial assay. Each vial has a droplet of SPLAT at the bottom, which functions as a test scent or OBP ligand dispenser. Note the clumped distribution of psyllid distribution within the vials.

Included in the test design were a negative control (citrus sprig + 100 μL blank SPLAT droplet) and a positive control where a citrus scent was added (citrus sprig + 100 μL SPLAT droplet with artificial citrus scent), since preliminary work indicated that the citrus scent was an attractant to *D. citri*. The composition of the artificial citrus scent ("Cmac" scent) was based on the most prevalent volatiles emitted from the young shoots of *C. macrophylla*. It contained a mixture of synthetic volatiles in the following proportions: 6.0 limonene: 3.6 citral: 1.2 β-ocimene: 1.0 β-caryophyllene: 1.0 terpinene: 0.6 linalool: 0.02 methyl salicylate.

The test with Ligand 937 was the first to be conducted. Its design included two concentrations of Cmac scent, 3- and 9 μL/10 mL SPLAT; these were compared to determine an optimal concentration of Cmac scent to use in tests with the other ligands. Since psyllid response was stronger to the 3-μL concentration of Cmac scent, only this concentration was used in subsequent tests.

In the Ligand 937 tests, there were seven treatments; each treatment vial was replicated twice in each incubator (14 vials/incubator). In the tests with ligands 019 and 654, there were six treatments; each treatment vial was replicated twice in each incubator (12 vials/incubator). In the tests with ligands 717, 784, 861, and 905, there were four treatments; each treatment was replicated four times (16 vials/incubator). Depending on the availability of sprigs and psyllids, three to four tests were conducted simultaneously in separate incubators for each 2-day experiment, resulting in 12–28 replicate experiments per ligand. Only a single ligand was tested simultaneously in the different incubators, and only a single ligand was tested during each complete experiment.

A separate test was conducted to evaluate psyllid settling levels in vials when no ligands were present. This test consisted of vials with a citrus sprig + a SPLAT droplet with Cmac scent and vials with a citrus sprig + a blank SPLAT droplet. A ratio of one Cmac vial: three blank SPLAT vials, with a total of 16 vials/incubator, was used to maintain a vial placement and density pattern that was similar to those used in the test ligands experiments.

**TABLE 1 |** Design of individual experiments showing treatments included in each, number of vials per treatment per each test, and the total number of vials used in each treatment in each experiment.

| Experiment | Treatments included in experiment | | | | | | | Number of vials per treatment per test | Total number of vials tested per treatment |
|---|---|---|---|---|---|---|---|---|---|
| | Blank SPLAT | 3 μL Cmac scent | 9 μL Cmac scent | 3 mg test ligand | 9 mg test ligand | 30 mg test ligand | 90 mg test ligand | | |
| No ligand | X | X | | | | | | 4 | 64 |
| 937 | X | X | X | X | X | X | X | 2 | 27 |
| 019 | X | X | | X | X | X | X | 2 | 40 |
| 654 | X | X | | X | X | X | X | 2 | 48 |
| 717 | X | X | | | X | X | | 4 | 48 |
| 784 | X | X | | | X | X | | 4 | 72 |
| 861 | X | X | | | X | X | | 4 | 60 |
| 905 | X | X | | | X | X | | 4 | 48 |

## Distribution Induces and Statistical Analysis

Data on psyllid distribution in incubators were analyzed as follows: each vial in the incubator was considered an experimental unit, or sample. Each incubator trial was considered a separate replicate. Mixed-model ANOVA was conducted for each test ligand, with treatment as a fixed effect and trial number (replicate) as a random effect (SAS version 9.4). *Post hoc* contrasts with a Bonferonni correction for multiple comparisons was conducted for all analyses with significant treatment effects using the least squares means test.

To ascertain the level of psyllid aggregation in the different vial treatments, the index corresponding to randomness ($D_P$), and the Morisita index ($I_m$) were calculated for each treatment (Hurlbert, 1990; Nansen et al., 2006; Mankin et al., 2014). The $D_P$ is based on the degree of overlap between the observed and a Poisson (random) distribution. The value of $D_P$ varies between 0 and 1, with 0 equal to 100% concordance with a Poisson distribution whereas values approaching 1 indicate an aggregated distribution (Hurlbert, 1990). The $I_m$ measured how many times more likely it is that two randomly selected psyllids will be from the same vial than it would be if the psyllids that settled during the 24-h period (the functional population) were distributed at random, with $I_m$ equal to 1.0 for random distributions (Hurlbert, 1990). These indices were then used as response variables in ANOVA tests comparing the ligands.

To determine the degree of the randomness in the distribution of psyllids within the different treatment vials, Poisson distributions were calculated for each treatment in each ligand test. The goodness of fit of the Poisson distribution to the observed data was then tested with the chi-square test (Zar, 1999).

As an additional measure of psyllid aggregation in each treatment, we determined the percentage of the total number of psyllids that arose from vials with ≥7 psyllids. A frequency of ≥7 psyllids per vial was selected as representing an aggregation because the presence of seven or more psyllids within a vial appeared to the observer to be definitively "crowded" (**Figure 1**). For each treatment within each test ligand experiment, the numbers of psyllids in vials with ≥7 psyllids were subjected to planned paired comparisons with the chi-square test (Zar, 1999) with $\alpha = 0.0083$. For these comparisons, the number of psyllids in vials with ≥7 psyllids in the blank SPLAT treatment was used as the expected frequency in the experiment-wide comparisons. The reason for using the blank SPLAT value as the expected frequency was that the frequency of psyllids that settled on citrus sprigs in control vials with no additional olfactory stimulus present within the vial was considered to represent a baseline level of settling behavior.

# RESULTS

The $D_p$ index shows the degree of overlap of the observed distribution with the Poisson distribution of the same population mean (Hurlbert, 1990). A $D_p = 0$ means that the population has a perfect Poisson distribution within the study area, while a $D_p = 1$ means that all the individuals are highly aggregated in a particular portion of the study area. In our study, the vials represented the study area and the values of the $D_P$ index ranged from 0.315 to 0.433. These values indicated that psyllid distributions in the vials were non-random in all cases (**Table 2**). $D_P$ values were not different among test ligands ($F_{7,139} = 2.26$; $P = 0.330$).

The $I_m$ index indicates how many times more likely it is that two randomly selected psyllids will be from the same vial than if they were randomly distributed. For example, if $I_m = 1.5$ then the probability of those two randomly selected individuals are from the same quadrat is 50% greater than it would be in the case of a random distribution. In the present study, $I_m$ values ranged from 1.33 to 1.93 (**Table 2**), showing that psyllids were 20–40% more clumped than they would be if their distributions were random. $I_m$ values among test ligands were different ($F_{7,139} = 2.43$; $P = 0.0225$), with the largest difference in $I_m$ values observed between test vials containing Ligand 019 test and the control test with no ligands (**Table 2**).

In the control test that evaluated psyllid settling levels in the absence of test ligands, similar numbers of psyllids settled in the Cmac scent treatment as in the blank SPLAT treatment (**Table 3** and **Figure 2**). Because psyllid settling levels were similar between the blank SPLAT and Cmac scent treatment, the addition of the Cmac scent did not appear to increase psyllid attraction *per se*.

**TABLE 2 |** Indices used to ascertain aggregation response of *D. citri* in settling vial assays.

| Ligand | $D_P$ index | $I_m$ index |
|---|---|---|
| None | 0.380 (0.03)[a] | 1.93 (0.18)[a] |
| 019 | 0.426 (0.03)[a] | 1.33 (0.06)[bc] |
| 654 | 0.433 (0.03)[ab] | 1.58 (0.08)[ac] |
| 717 | 0.366 (0.04)[a] | 1.50 (0.13)[ac] |
| 784 | 0.391 (0.03)[a] | 1.53 (0.10)[ac] |
| 861 | 0.341 (0.03)[a] | 1.53 (0.10)[ac] |
| 905 | 0.385 (0.04)[a] | 1.47 (0.15)[ac] |
| 937 | 0.315 (0.02)[ac] | 1.46 (0.07)[bc] |

*$D_P$, index corresponding to randomness; $I_m$, Morisita index. Values shown are mean ± SEM. Means with different letters in the same column are different, $P \leq 0.03$.*

**TABLE 3 |** Comparison of the mean numbers of psyllids per vial between treatments within each ligand test.

| Ligand | *F* value | *P*-value |
|---|---|---|
| None | 0.11 | 0.7418 |
| 019 | 1.6 | 0.1631 |
| 654 | 4.86 | 0.0003 |
| 717 | 11.19 | <0.0001 |
| 784 | 16.01 | <0.0001 |
| 861 | 5.94 | 0.0007 |
| 905 | 0.45 | 0.7174 |
| 937 | 2.13 | 0.0493 |

*The F values were obtained by ANOVA; the P-value is shown in the right hand column.*

However, the Cmac scent treatment had a greater percentage of psyllids from vials with ≥7 psyllids than did the vials with blank SPLAT (**Figure 3**), suggesting that the presence of Cmac scent had some influence on psyllid aggregation behavior. Neither of the observed distributions in blank SPLAT control and Cmac scent vials fit the Poisson distributions (**Figure A1** in **Supplementary Material**), demonstrating that the distributions in both treatments were non-random.

The mean number of psyllids per vial varied between treatments in all of the ligand tests except for Ligands 019 and 905 (**Table 3**). In tests with Ligands 717, 784, and 861, vials with Cmac scent had greater numbers of psyllids per vial than in the other treatment vials (**Figure 2**). In Ligand 654 test, the settling level was higher in the Cmac scent treatment than in the blank SPLAT and 30 mg ligand concentration treatments. In tests with Ligands 654, 717, 784, and 861, the percentage of psyllids from vials with ≥7 psyllids was also greatest in the vials with Cmac scent (**Figure 3**). In the Ligand 784 and 861 tests, none of the observed distributions in the individual treatments matched the Poisson distribution (**Figures A2, A3** in **Supplementary Material**). In the Ligand 717 test, the observed distribution in the Cmac scent treatment did not fit the Poisson distribution while those in the blank SPLAT, 9 and 30 mg ligand treatments were similar to the Poisson distribution (**Figure A4** in **Supplementary Material**). In the Ligand 654 test, only the observed distribution in the 30 mg ligand treatment fit the Poisson distribution (**Figure A5** in **Supplementary Material**).

There was no difference in the mean number of psyllids/vial among the treatments in the tests with ligands 019, 905, and 937 (**Table 3**). In the Ligand 019 test, numerically fewer psyllids settled in the vials with Cmac scent (**Figure 2**), while the Cmac scent treatment in this experiment also had the lowest frequency of vials with ≥7 psyllids (**Figure 3**). Only the observed distribution in the Cmac scent treatment matched the Poisson distribution (**Figure A6** in **Supplementary Material**). In the tests with ligands 905 and 937, similar numbers of psyllids settled in the Cmac and blank SPLAT treatment (**Figure 2**). However, in both ligands, the Cmac scent treatment had a higher percentage of psyllid from vials with ≥7 psyllids than did the blank SPLAT treatment (**Figure 3**); and none of observed distributions fit the Poisson distribution (**Figures A7, A8** in **Supplementary Material**).

# DISCUSSION

## Olfactory Stimuli and Psyllid Aggregation

Since the psyllids were reared on *C. macrophylla*, it was expected that the Cmac scent would function as an attractant and lure psyllids into the test vials. However, because the settling levels of psyllids were similar in the blank SPLAT and Cmac scent vials, it appears that the Cmac scent did not function as an attractant, i.e., it did not lure more psyllids into the vials with Cmac scent. On the other hand, psyllid aggregation levels were slightly higher in the vials with Cmac scent, indicating that it did have a modest effect on the psyllids' aggregation behavior. In a previous study that used a settling vial assay, similar results were observed in which the mean numbers of psyllids per vial were similar among control and treatment vials but enhanced psyllid aggregation levels were observed in treatment vials (Patt et al., 2018). The results of these studies with settling vial assays suggest that, while the mean number of psyllids per vial is the primary indicator of psyllid response to a particular test odorant, aggregation level is also an important indicator of a test odorant's biological activity. The values of the aggregation indices showed that psyllid distribution was clumped in most of the ligand tests. Comparison of the observed frequency distributions with the Poisson distribution also demonstrated that, in most cases, the psyllids' distribution was non-random. The distribution of *D. citri* within citrus trees and groves is aggregated (Tsai et al., 2000; Sétamou et al., 2008; Costa et al., 2010; Hall and Hentz, 2010). Because multiple matings are apparently required for high reproductive output by female psyllids (Wenninger and Hall, 2007), aggregation behavior may play a central role in *D. citri* reproduction. If this is true, then olfactory stimuli that promote aggregation behavior may be an important means of increasing the efficacy of traps or attract and kill devices because they would promote psyllid congregation.

## OBP Ligands Modulating the Response of *D. citri* Behavior in Test

Four of the test ligands (654, 717, 784, and 861) enhanced the response of *D. citri* to the Cmac scent mixture both in terms

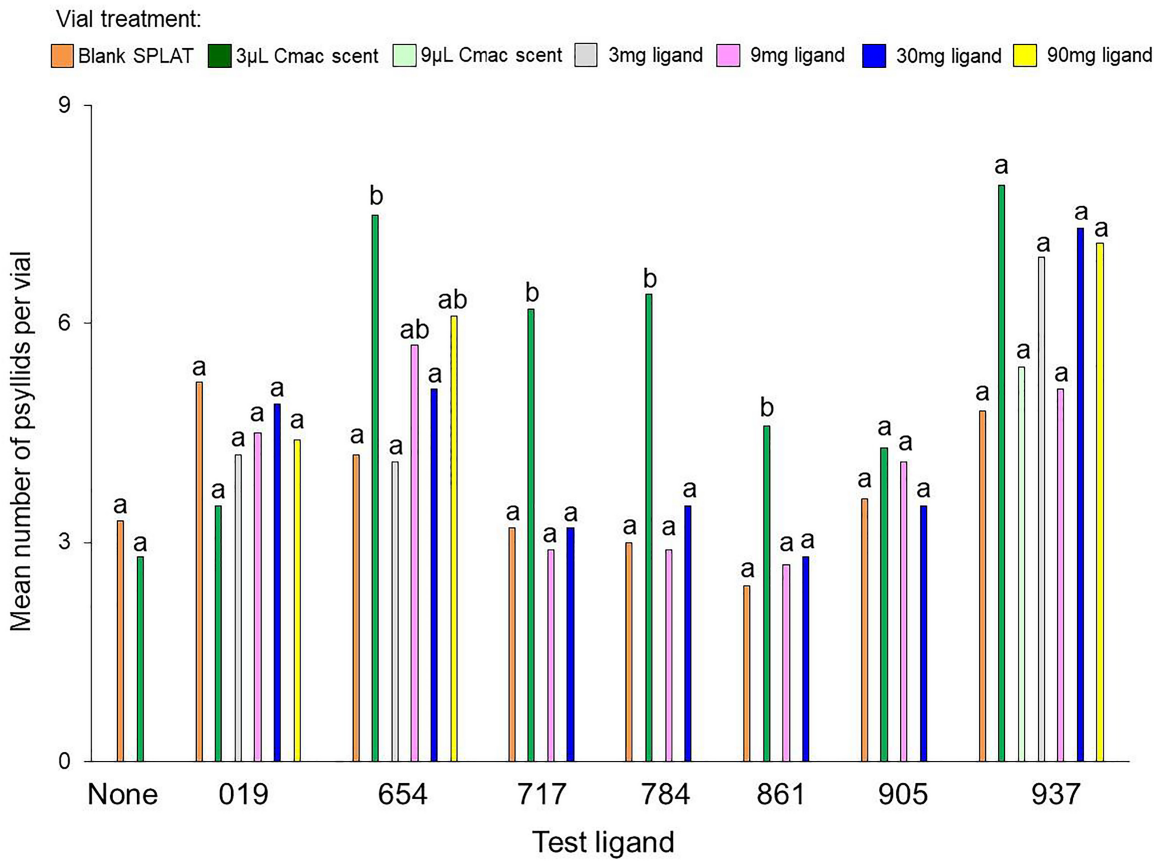


**FIGURE 2 |** Mean number of psyllids per vial in each separate experiment with the test ligands and the blank SPLAT control. Bars with different letters within the same test ligand are different at $P \leq 0.05$.

of attractiveness and aggregation level. These results concurred with those of Patt et al. (2013), who found that psyllid probing increased when a synthetic OBP ligand was combined with a single citrus volatile, and with Aksenov et al. (2014), who observed enhanced *in vitro* responses when OBP ligands were presented with citrus volatiles. Interestingly, the antennal OBPs tested by Aksenov et al. (2014) reacted only to mixtures of citrus volatiles, not to individual volatiles. Exposure to test ligands 654, 717, 784, and 861 may have made the psyllids more receptive to the Cmac scent, and possibly to other close-range chemosensory, visual, and vibrational stimuli used by the psyllids to select host plants and located conspecifics. Another possibility is that, once the psyllids perceived these test ligands, they became stimulated to follow the concentration gradient of Cmac scent emanating from the vials.

## Ligand Inhibiting the Response of *D. citri*

In the Ligand 019 experiment, the Cmac scent treatment vials had numerically the fewest psyllids while the blank SPLAT vials had high aggregation levels. These results indicated that Ligand 019 either interfered with or inhibited the attraction and aggregation responses of *D. citri* to the Cmac scent. Since numerically more psyllids settled in vials with Ligand 019 than in Cmac scent, the ligand itself did not appear to be a repellent to *D. citri*; nonetheless, it influenced the behavioral response of the psyllids to the Cmac scent.

## OBP Ligands With No Apparent Effects on *D. citri*

Although there were overall treatment effects in the experiment with Ligand 937, there were no significant differences in psyllid settling levels between individual treatments. A numerically greater number of psyllids settling in the Cmac scent treatment vials than in the vials with blank SPLAT and the percentage of vials with ≥7 psyllids was significantly greater in vials with Cmac scent versus blank SPLAT. Whereas Ligand 937 induced high levels of probing, its effects on psyllid settling or aggregation behavior were not as obvious. Ligand 905 had no observable effect on psyllid settling response but the percentage of vials with ≥7 psyllids was significantly greater in vials with either Cmac scent or 9 mg Ligand 905 versus blank SPLAT.

## Conclusion and Possible Application

In the present study, the simultaneous presentation of a synthetic OBP ligand and a mixture of citrus volatiles influenced two behaviors in *D. citri*, namely, host plant attraction and

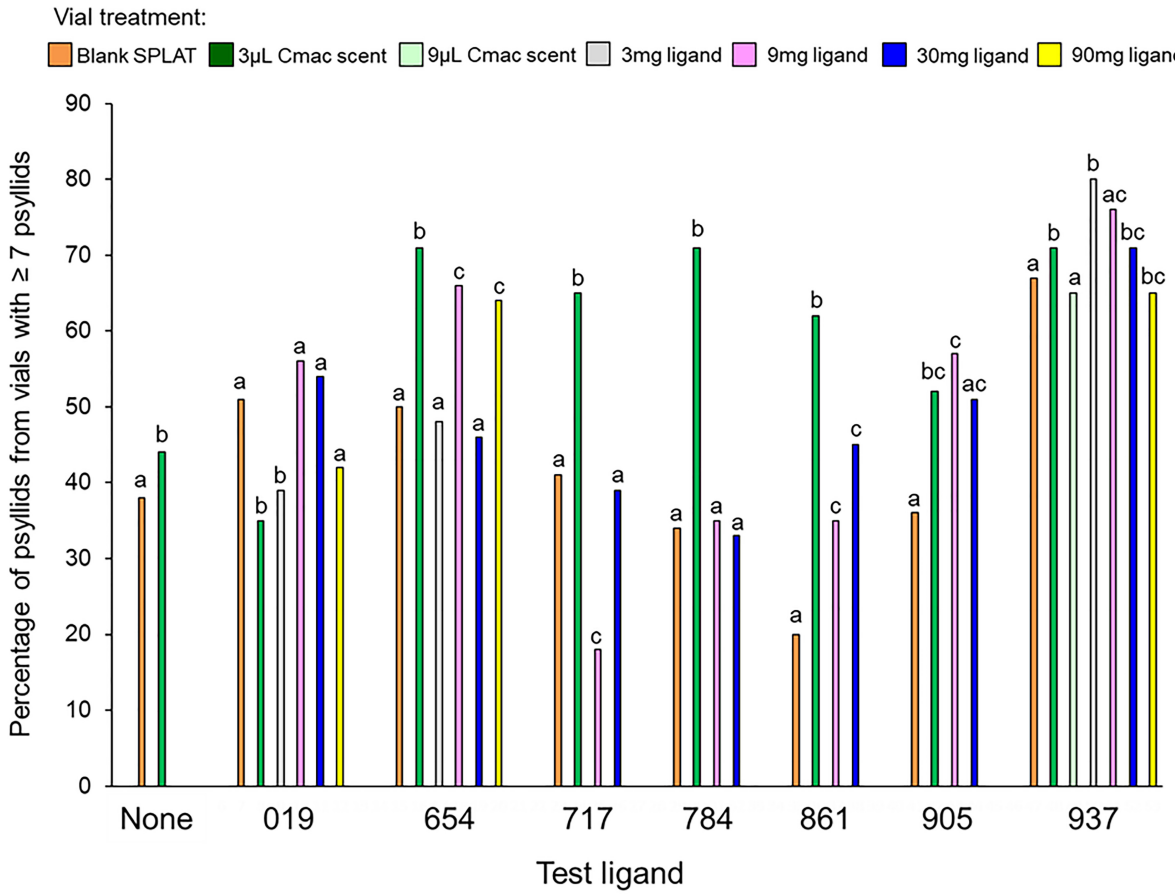


**FIGURE 3 |** Percentage of psyllids from vials with ≥7 psyllids in each treatment. Bars with different letters within the same test ligand group are different at $P \leq 0.05$.

aggregation on host plants. It seems likely that the psyllids perceived a test ligand and the Cmac scent volatiles within the confines of the incubators prior to their entry into the vials. Within the incubators, psyllids frequently moved up and down from the overhead lights to the vial caps; this would have given them the opportunity to come into contact with test ligand and the Cmac scent while outside the vials.

Interestingly, increased settling and aggregation levels were largely not observed in the vials with the test ligand treatments, and there was little effect of ligand concentration on psyllid response levels. This gives credence to the notions that the psyllids perceived the test ligands while in the incubator and that the test ligands themselves did not attract the psyllids. While it may seem intuitive that the psyllids perceived the ligands and citrus volatiles outside of the vials, our study did not include an evaluation of volatile emission levels from the SPLAT droplets, at the vial entrance holes, or within the incubator atmosphere. Therefore, we did not have empirical evidence that behaviorally meaningful ligand or citrus volatile concentrations were present outside of the vials, i.e., in the chamber atmosphere. In fact, our *a priori* assumption was that the greatest level of olfactory stimulation occurred when the psyllids perched at the entrance holes of the vials, where they would have been exposed to ligand concentrations representative of the vial interiors rather than the chamber atmosphere. We conjectured that the combination of visual and olfactory stimuli presented to the psyllids at the entrance holes would cause them to enter the vials. The results obtained instead suggest that it is more likely that the psyllids perceived an "average" level of ligand in the chamber atmosphere, which would have been a function of the total amount of volatilized ligand emitted from all of the ligand treatment vials, and once they perceived the ligand, they responded more strongly to the Cmac volatiles. Further studies using electrophysiological assays, the response of DCSAP4 and other psyllid OBPs to the different ligands, and/or more refined behavioral assays are needed to determine whether psyllids respond to the ligand and citrus volatiles concomitantly or if perception of the ligand increases psyllid response to the citrus volatiles. Lastly, if some of the ligands had low volatility, it may help explain why there was no behavioral effect observed in experiments where they were presented to the psyllids. This is another area for further investigation.

It is also curious that many vials had no psyllids in them, since each contained a young citrus shoot and these were expected to be highly attractive themselves to the psyllids. The numerous vials observed with no or only a single psyllid in them also

gives support to the idea that a combinational or synergistic effect between the test ligand and Cmac scent occurred and that this interaction was an initial step leading to the development of aggregations within vials with Cmac scent. The synthetic ligands tested in this study could be good candidates to further examine the molecular underpinnings of *D. citri* perception of chemosensory cues.

In terms of application, these results indicate that the addition of some of these test ligands to mixtures of citrus volatiles could significantly promote the effectiveness of these mixtures as scent lures for *D. citri* in conjunction with monitoring traps or attract and kill devices. Ligand 019 might be effective at repelling psyllids. However, further work is needed with respect to evaluating the toxicology of the ligands and ensuring that they are safe to release in the environment, whether their performance in the field significantly elevates psyllid capture levels, and whether the additional cost of adding them to scent lures or trapping devices is justified in terms of enhanced psyllid capture level.

## AUTHOR CONTRIBUTIONS

JP invented the settling assay, designed the experiments, supervised the execution of the project, conducted some of the statistical analysis, and wrote the manuscript. WM conducted the statistical analyses in the main, contributed to the "Statistical Analysis" and "Results" sections of the manuscript, and contributed to the development of the manuscript. RN contributed to the experimental design and statistical analyses. DW provided the synthetic ligands and contributed to the experimental design.

## FUNDING

This research was funded by USDA Agricultural Research Service and a USDA SBRI grant to Inscent, Inc. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation of endorsement by the USDA for its use.

## ACKNOWLEDGMENTS

We are grateful to A. Moreno, N. Gaza, A-S Hill, and F. Manthey for their assistance with rearing the psyllids and conducting the experiments, to ISCA Technology for providing SPLAT, and to R. Shatters and P. Robbins for reviewing a draft of the manuscript.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01891/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

REVIEW
published: 22 January 2019
doi: 10.3389/fpls.2018.01976

# Effect of Huanglongbing or Greening Disease on Orange Juice Quality, a Review

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**Specialty section:**
*This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Plant Science*

**Received:** *20 July 2018*
**Accepted:** *19 December 2018*
**Published:** *22 January 2019*

**Citation:**
*Dala-Paula BM, Plotto A, Bai J, Manthey JA, Baldwin EA, Ferrarezi RS and Gloria MBA (2019) Effect of Huanglongbing or Greening Disease on Orange Juice Quality, a Review. Front. Plant Sci. 9:1976. doi: 10.3389/fpls.2018.01976*

Huanglongbing (HLB) or citrus greening is the most severe citrus disease, currently devastating the citrus industry worldwide. The presumed causal bacterial agent *Candidatus* Liberibacter spp. affects tree health as well as fruit development, ripening and quality of citrus fruits and juice. Fruit from infected orange trees can be either symptomatic or asymptomatic. Symptomatic oranges are small, asymmetrical and greener than healthy fruit. Furthermore, symptomatic oranges show higher titratable acidity and lower soluble solids, solids/acids ratio, total sugars, and malic acid levels. Among flavor volatiles, ethyl butanoate, valencene, decanal and other ethyl esters are lower, but many monoterpenes are higher in symptomatic fruit compared to healthy and asymptomatic fruit. The disease also causes an increase in secondary metabolites in the orange peel and pulp, including hydroxycinnamic acids, limonin, nomilin, narirutin, and hesperidin. Resulting from these chemical changes, juice made from symptomatic fruit is described as distinctly bitter, sour, salty/umami, metallic, musty, and lacking in sweetness and fruity/orange flavor. Those effects are reported in both Valencia and Hamlin oranges, two cultivars that are commercially processed for juice in Florida. The changes in the juice are reflective of a decrease in quality of the fresh fruit, although not all fresh fruit varieties have been tested. Earlier research showed that HLB-induced off-flavor was not detectable in juice made with up to 25% symptomatic fruit in healthy juice, by chemical or sensory analysis. However, a blend with a higher proportion of symptomatic juice would present a detectable and recognizable off flavor. In some production regions, such as Florida in the United States, it is increasingly difficult to find fruit not showing HLB symptoms. This review analyzes and discusses the effects of HLB on orange juice quality in order to help the citrus industry manage the quality of orange juice, and guide future research needs.

**Keywords:** ***Candidatus*** **Liberibacter asiaticus, Valencia, Hamlin, flavor, bitter compounds**

## INTRODUCTION

Huanglongbing (HLB) is a citrus disease that has profoundly changed the size and shape of worldwide citrus production, and the negative effects keep impacting the industry as the disease continues to spread throughout the various citrus growing regions of the world (Gottwald et al., 2012). Practically all commercial citrus species and cultivars are vulnerable to HLB. The disease

has an array of symptoms which can be detected anywhere on the plant, from the roots to the leaves, changing the chemical characteristics, and sensory attributes of the fruit (Bové, 2006; Baldwin et al., 2010, 2018; Dala Paula et al., 2018). In this review, the effects of HLB on orange juice quality are described based on the current scientific literature.

## WORLDWIDE CONSUMPTION AND PRODUCTION OF FRESH ORANGES AND ORANGE JUICE

Orange juice is the most widely consumed fruit juice in the world (Markestrat., 2016). Brazil is the world's largest orange producer and is forecasted to reach production levels of ∼17.3 million tons for the 2017/2018 season. China is foreseen to be the second largest producer with 7.3 million tons followed by the European Union−6.3 million tons, the United States (US)−3.6 million tons, and Egypt−3.2 million tons (USDA-FAS Foreign Agricultural Service, 2018a). For the 2018–2019 season, Brazilian commercial orange production is predicted to decrease 27% due to high temperatures in October 2017 and stress from the previous production cycle (USDA-FAS Foreign Agricultural Service, 2018b). American commercial orange production is projected to drop 23% due to several reasons including the damage by Hurricane Irma in September 2017 added to the presence of HLB in Florida, and unfavorably hot weather in California (USDA-FAS Foreign Agricultural Service, 2018a).

Currently, citrus producers in many countries are facing serious problems with the emergence of the HLB disease (Teixeira et al., 2008; Bassanezi et al., 2009, 2011; Spreen and Zansler, 2015). HLB was responsible for the decrease in the production of oranges for processing in the United States from 7.98 to 2.22 billion tons (72.2% reduction) from 2007–08 to 2017–18. The fresh fruit market also decreased from 2.10 to 1.70 billion tons (20.5% reduction) during the same time interval. This market was less impacted than the rest of the citrus industry because, in the United States, around 90% of the oranges produced in Florida, the state with the largest prevalence of HLB, are processed while California supplies oranges for the fresh market (USDA-NASS, 2018). Singerman et al. (2017) reported an increase from $2.89 to $9.34 (3.2 times) of the price of a box of orange since HLB had been detected in the United States.

## A BRIEF HISTORICAL BACKGROUND OF HUANGLONGBING OUTBREAKS

Huanglongbing means "yellow dragon disease" in Chinese, and is also known as citrus greening (Halbert and Manjunath, 2004). HLB is considered one of the most severe citrus diseases in the world and, consequently, a serious problem for the citrus processing industry. The disease affects nearly all varieties of citrus, with grapefruit, sweet oranges, some tangelos, and mandarins being the most susceptible and limes, lemons, sour oranges, and trifoliate oranges the least (Abdullah et al., 2009).

It is difficult to determine where HLB originated. However, there is information suggesting that HLB was responsible for India's citrus dieback during the eighteenth century (Capoor, 1963; da Graça, 2008). Initially, researchers believed that the tristeza virus was the leading cause of the citrus dieback in India, but after a thorough survey, HLB was determined to be the primary cause (Fraser and Singh, 1968; da Graça, 2008). In China, HLB has been reported since 1919 and described by Reinking (1919) as the citrus yellow shoot disease (Bové, 2006). In 1937, the African variation was reported for the first time in South Africa (Van der Merwe and Andersen, 1937), and it was later linked to chromium and manganese toxicity. It was also associated with the leaf mottling citrus disease in the Philippines in the 1960's (Fraser et al., 1966; McClean and Schwarz, 1970). Currently, the disease has spread to more than 50 countries in Africa, Asia, Oceania, and the Americas (South, North and Central Americas, and the Caribbean; **Figure 1**; CABI, 2017; EPPO, 2017).

The first case of HLB in the Americas was reported in the state of São Paulo, Brazil in 2004 (Coletta-Filho et al., 2004; Teixeira et al., 2005a). However, in a survey conducted in São Paulo, just 6 months after HLB had been reported in Brazil, 46 cities stated having infected trees, suggesting that HLB had been present for almost 10 years (Bové, 2006). A year later, in August 2005, symptoms of the disease were recognized in Florida, United States; in 2007 in Cuba; in 2008 in the Dominican Republic; and in 2010 in Mexico (Coletta-Filho et al., 2004; Halbert, 2005; Llauger et al., 2008; Matos et al., 2009; NAPPO North American Plant Protection Organization, 2010). Currently, HLB is present in all Florida citrus-growing counties (Baldwin et al., 2010), in California, Georgia, Louisiana, South Carolina, and Texas (CABI, 2017; EPPO, 2017). As the severity of HLB increases, premature fruit drop becomes a growing problem which has contributed to declining yields in Florida, especially during the last few years (Chen et al., 2016). In Brazil, the States of São Paulo, Minas Gerais, and Paraná have reported the presence of HLB, with São Paulo being the most affected state. In India and China, HLB has spread to around 25 and 11 provinces, respectively (**Table S** in Supplementary Material; CABI, 2017; EPPO, 2017).

## CAUSAL AGENTS AND VECTORS OF HUANGLONGBING

It is well established that Huanglongbing is associated with the presence of the gram-negative bacteria genus *Candidatus* Liberibacter (*C*L). Three species are known to cause the symptoms of HLB: *C*L asiaticus (*C*Las), *C*L americanus (*C*Lam), and *C*L africanus (*C*Laf). The Asian and the American species can be transmitted by the psyllid *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), commonly called Asian citrus psyllid (ACP), and the African species by the insect *Trioza erytreae* (Hemiptera: Triozidae; Bové, 2006). Although HLB was first

**Abbreviations:** AS, Asymptomatic; *C*L, *Candidatus* Liberibacter; *C*Laf, *Candidatus* Liberibacter africanus; *C*Lam, *Candidatus* Liberibacter americanus; *C*Las, *Candidatus* Liberibacter asiaticus; DNA, Deoxyribonucleic acid; FJ, Filtered juice; HLB, Huanglongbing; JWP, Juice with pulp; MT, Metric tons; PCR, Polymerase chain reaction; rDNA, Recombinant Deoxyribonucleic acid; SSC, Soluble solids content; SY, Symptomatic; TA, Total acidity.

**FIGURE 1 |** Countries currently affected by Huanglongbing (HLB) disease (adapted from CABI, 2017; EPPO, 2017).

reported in Brazil and the US 15 years ago, the psyllid vector was reported in São Paulo and Florida as early as 1942 and 1998, respectively (Bové, 2006; Tansey et al., 2017).

*C*Lam was the most prevalent bacteria species in Brazil in 2005, which initially affected more than 90% of the infected trees, decreasing to 60% in 2007. During this period, there was an increase in *C*Las infection, from 5 to 35% of the infected trees, while a combined infection remained practically the same at 5% (Coletta-Filho et al., 2007; Gasparoto et al., 2012). Among HLB bacteria, *C*Laf is sensitive to heat and to dry weather and thrives between 20 and 25°C, while the other species are heat tolerant and thrive at higher temperatures (Catling, 1969; Cheraghian, 2013). These observations might explain why *C*Laf is not present in hot and humid tropical and subtropical climates.

As *C*Las has been difficult to culture *in vitro*, its recommended detection methods was by quantitative real-time polymerase chain reaction (qPCR) targeting the 16S rDNA gene (Teixeira et al., 2005b; Li et al., 2006).

## SYMPTOMS OF HUANGLONGBING AND ITS IMPACT ON ORANGE TREES

In the early stages of the disease, it is difficult to make a clear diagnosis. McCollum and Baldwin (2017) noted that HLB symptoms are more apparent during cooler seasons, more so than in warmer months. It is uncertain how long a tree can be infected before showing the symptoms of the disease but, when it eventually becomes symptomatic, symptoms appear on different parts of the tree. Infected trees generally develop some canopy thinning, with twig dieback and discolored leaves, which appear in contrast to the other healthy or symptomless parts of the tree. The symptomatic leaves can be normal-sized, showing yellow coloration or a blotchy-mottle or they can be small, upright and show a variety of chlorotic patterns resembling those induced by zinc or other nutritional deficiencies (McClean and Schwarz, 1970; da Graça, 1991; Albrecht et al., 2016; McCollum and Baldwin, 2017).The root systems are poorly developed, showing very few fibrous roots, likely due to nutrient starvation (da Graça, 1991; Batool et al., 2007).

Symptomatic trees display excessive starch accumulation in the aerial plant parts, one of the predominant biochemical responses to HLB, due to the upregulation of glucose-phosphate transport, which is involved with the increased entrance of glucose into this pathway (Martinelli and Dandekar, 2017). It has been suggested that accumulation of starch in the leaves is also the result of decreased degradation and impaired transport which results in an inefficient partitioning of photoassimilates among mature citrus leaves, roots, and young leaves. This unbalance in sugar transport and accumulation would affect sugar content in fruit. The starch indefinitely remains in the aerial plant parts; it does not degrade, even during the night cycles, resulting in root starvation, severe health decline, and death of trees (Etxeberria et al., 2009; Fan et al., 2010; Zheng et al., 2018).

Along with the color changes and starch accumulation in symptomatic leaves, there are also changes in the secondary metabolite profiles. HLB affects the amounts of hydroxycinnamic acids and flavonoids in infected leaves, resulting in lower levels of vicenin-2, apigenin-*C*-glucosyl-*O*-xyloside, 2”-xylosylvitexin, luteolin rutinoside, and isorhoifolin compared to healthy leaves. While healthy leaves contain only trace levels of limonin glucoside, infected leaves contain levels of 300 $\pm$ 22 $\mu$g/mL (Manthey, 2008). Proline and other amino-acids were found in greater amounts in leaves showing symptoms of infection, and sugar metabolism was also affected (Cevallos-Cevallos et al., 2012; Albrecht et al., 2016).

According to studies of infected orange fruit, HLB-symptomatic oranges are reduced in size, sometimes asymmetric, and contain small, brownish/black aborted seeds which can be seen when the orange is sectioned perpendicularly to the fruit axis. The orange peel turns green with an inversion of colors: the fruit turns from green to yellow/orange in the peduncular end while the stylar end remains green. In a healthy orange, the color change first starts at the stylar end, progressing only later to the peduncular area. HLB causes fruits to drop prematurely, resulting in a 30–100% yield reduction, and, ultimately, premature death of the tree. Tree mortality can occur several months to years after infection (McClean and Schwarz, 1970; da Graça, 1991; Bové, 2006; Batool et al., 2007; Bassanezi et al., 2011; Liao and Burns, 2012).

HLB symptomatic fruit from infected trees are smaller in diameter compared to asymptomatic fruit from infected and healthy trees, which have similar diameter (**Table 1**, **Figure 2**). Even though most of these symptomatic fruit do not make it to processing due to premature drop or elimination by sizing equipment (McCollum and Baldwin, 2017; Baldwin et al., 2018), more are entering the processing stream as there is not enough normal sized fruit. The weight and juice content of symptomatic oranges are diminished compared to asymptomatic and healthy oranges, which are similar (**Table 1**). Most of the studies were performed with Valencia and Hamlin oranges (Liao and Burns, 2012; Massenti et al., 2016; Baldwin et al., 2018), and also with two strains of Valencia, and Hamlin, Westin and Pera varieties (Bassanezi et al., 2009).

HLB potentially causes trees to be more susceptible to other pests including citrus longhorned beetle (*Anoplophora chinensis* Forster) attacks. In advanced cases of HLB infection, a combination of citrus longhorned beetles and *Phytophthora* fungi is common (Halbert and Manjunath, 2004; Batool et al., 2007).

## HUANGLONGBING CONTROL AND MITIGATION OF ITS SYMPTOMS

Current management strategies focus on vector control, avoiding the spread of infection, or management of infected trees. The success of individual or combined approaches depends on the infestation level. In regions where disease incidence is low, the most common practices are avoiding the spread of infection by removal of symptomatic trees, protecting grove edges through intensive monitoring, use of pesticides, and biological control of the vector ACP. The management of infected trees includes enhanced nutrition by foliar sprays of readily absorbable nutrients and phytohormones, or regulating soil pH to enhance nutrient uptake, and precision irrigation based on soil moisture sensing and needs of HLB-affected trees (Stansly et al., 2010; Albrecht et al., 2012; Martini et al., 2016; Zheng et al., 2018). However, the control of HLB is still difficult, especially if bacteria are widespread and their vectors are well established. Diseased trees in abandoned citrus groves act as abundant sources of CLas inoculation and insect vectors, and this has been a particularly prevalent problem in Florida. The most effective control strategy has been to remove infected trees in an area and then replant with CLas-free trees (Abdullah et al., 2009). Current recommendations are that control of the psyllid vector should be done as soon as its presence is noticed in citrus groves, even in regions free of HLB (McCollum and Baldwin, 2017).

Another area-wide pest management approach to control the ACP and reduce the likelihood of resistance is the Citrus Health Management Areas (CHMAs) (Jones et al., 2013). According to Singerman and Useche (2016), CHMAs coordinate insecticide application to control the ACP spreading across area-wide neighboring commercial citrus groves as part of a plan to address the HLB disease. The intensifying insecticide application also creates environmental and public health concerns and side-effects to specific fauna, as the arthropod (Monzo and Stansly, 2017). Singerman and Page (2016) indicated that CHMAs enhance grower's profitability when all growers involved participated in the program.

Covered, protected production fields have been tested as an alternative for fresh citrus production in Florida. These protected systems work by physically excluding ACP from the enclosed grove therefore preventing contact between the ACP and trees. One of the main advantages is the reduced reliance upon frequent insecticide sprays to control psyllids (Ferrarezi et al., 2017a). Anti-psyllid screen houses and container-grown cultivation allow rapid young plant growth, thus playing important roles in developing new citrus production systems aimed at vector-free environments (Ferrarezi et al., 2017b).

Florida growers have been using foliar nutritional spray products that often contain macro- and micro-nutrients to compensate for lack of nutrient assimilation due to the disease, and compounds that are believed to activate "systemic acquired resistance" pathways in plants (such as salicylic acid) to increase tree defense response (Masuoka et al., 2011; Baldwin et al., 2012a). The benefits of this approach to disease management in the field have been criticized because the inoculum remains after application. Unfortunately, this perceived method of managing HLB potentially contributed to the proliferation of the disease in Florida after farmers stopped eliminating their infected trees. Unless the vector is thoroughly controlled, the spread of HLB to other orchard trees and neighboring farms is inevitable (Timmer et al., 2011; Gottwald et al., 2012). In an evaluation of the effect of nutritional spray treatments on fruit quality, Hamlin oranges from treated trees had the same off-flavor as oranges from trees that did not receive the treatment, whereas Valencia oranges were notably sweeter. Nutritional treatments did not consistently result in less pathogen DNA for either variety (Baldwin et al., 2012a). The implementation of combined nutrient programs and insecticide treatments has been studied and the results suggest that the beneficial effect of increased orange juice quality may have been cumulative, only manifesting later on (Baldwin et al., 2017; Plotto et al., 2017).

In addition to foliar nutritional sprays, plant growth regulators were tested, unsuccessfully, to reduce HLB-associated fruit drop (Albrigo and Stover, 2015). Incidentally, it was found that orange fruit showing HLB symptoms were also contaminated with *Lasiodiplodia theobromae* (diplodia), generally a postharvest pathogen, but which induced greater abscission zone in

**TABLE 1 |** Changes in diameter, weight, and juice content of fruit affected by Huanglongbing.

| References | Orange sample | | Fruit parameters | | |
|---|---|---|---|---|---|
| | Harvest time | Status or conditions | Diameter (cm) | Weight (g) | Juice (g/100 g) |
| **VALENCIA ORANGE JUICE** | | | | | |
| Bassanezi et al., 2009[I] | Blend of different harvests[1] | HLB-AS | 7.3a | 208.1a | 50.0a |
| | | HLB-SY | 5.9b | 118.9b | 44.6b |
| Liao and Burns, 2012[II] | April 2009 | Healthy | 7.4a | 208.5a | 53.2a |
| | | HLB-AS | 7.7 a | 214.5 a | 52.9a |
| | | HLB-SY | 5.8b | 122.3 b | 46.1b |
| Massenti et al., 2016[III] | March and May 2013[2] | Healthy | – | 183b | 58.9a |
| | | HLB-AS | | 208a | 57.8ab |
| | | HLB-SY | | 115c | 55.5b |
| Baldwin et al., 2018[IV] | April 2015 | Healthy | 6.7ab | – | – |
| | | Healthy-R | 6.9a | | |
| | | Healthy-D | 7.0a | | |
| | | HLB-SY-R | 6.2c | | |
| | | HLB-SY-D | 6.4bc | | |
| **HAMLIN ORANGE JUICE** | | | | | |
| Bassanezi et al., 2009[I] | Blend of different harvests[3] | HLB-AS | 6.9 a | 173.1 a | 42.2a |
| | | HLB-SY | 6.1b | 128.6b | 39.3b |
| Liao and Burns, 2012[II] | December 2007 | Healthy | 7.2a | 194.3a | 52.1a |
| | | HLB-AS | 6.9a | 196.6a | 49.9a |
| | | HLB-SY | 5.3b | 109.9b | 48.8a |
| Baldwin et al., 2018[IV] | December 2014 | Healthy | 6.8abc | – | – |
| | | Healthy-R | 7.1ab | | |
| | | Healthy-D | 7.3 a | | |
| | | HLB-SY-R | 6.7bc | | |
| | | HLB-SY-D | 6.4c | | |
| | January 2015 | Healthy | 6.9a | | |
| | | Healthy-R | 6.9a | | |
| | | Healthy-D | 6.9a | | |
| | | HLB-SY-R | 6.1b | | |
| | | HLB-SY-D | 6.2b | | |

*AS, asymptomatic; SY, symptomatic; Healthy, fruit harvest from healthy not shaken trees; Healthy-R, fruit harvest from healthy shaken trees (healthy-retain); Healthy-D, healthy fruit that dropped to the ground upon shaking the trees (healthy-drop); HLB-R, fruit retained on shaken HLB affected-trees; HLB-D, fruit that dropped from HLB affected-trees.*

[1]*Blend of oranges harvested on September 2004, July and October 2005 and August 2007;*

[2]*Blend of oranges harvested on March and May 2013;*

[3]*Blend of oranges harvested on July 2007, June and July 2008.*

*Values from the same reference with the same letter within columns and same harvest time are not significantly by the following statistical analysis ([I]Student t-test with the probability of error estimated to be lower than 0.000; [II]Duncan's multiple range test $p \leq 0.01$; [III]Tukey's test at $p \leq 0.05$; [IV]ANOVA and Tukey's test at $p \leq 0.05$).*

symptomatic fruit (Zhao et al., 2015). A direct correlation between diplodia and ethylene production at the fruit abscission zone was established, and the use of pre-harvest fungicides reduced fruit drop (Zhao et al., 2016). However, HLB-infected fruit with a greater abscission zone (i.e., fruit that are more readily prone to drop on the ground) had generally lower quality than fruit harvested from the same trees but with lesser abscission zone (Baldwin et al., 2018). The difference in quality was due to lower total sugars and high bitter limonoids, and was more pronounced in early-harvested Hamlin. The strategy of reducing fruit drop by reducing diplodia infection might have its benefit in delaying harvest to reduce the negative effect of HLB on fruit quality.

## FRESH FRUIT AND ORANGE JUICE QUALITY AFFECTED BY *CANDIDATUS* LIBERIBACTER ASIATICUS

To better understand the influence of HLB on the chemical and physicochemical characteristics of orange juice, it is important to consider the factors which may affect them, such as variety, harvest date, location, maturity, and the presence of pulp in the juice. In general, variations due to harvest date are more pronounced compared to variation due to the disease (Bassanezi et al., 2009; Baldwin et al., 2010; Plotto et al., 2010). As the season progresses, the peel color of a healthy orange becomes less green and more orange, juice content


**FIGURE 2 |** Size and color differences between fruit affected by Huanglongbing (HLB). **(A)** Hamlin healthy; **(B)** Hamlin HLB-asymptomatic; **(C)** Hamlin HLB-symptomatic; **(D)** Valencia healthy; **(E)** Valencia HLB-symptomatic (Photography by the authors).

declines, sugars and soluble solids content (SSC) increase and titratable acidity (TA) and citric acid decrease (Bai et al., 2016).

## Sensory and Chemical Composition Changes of HLB-Affected Fruit

### Peel Color

As peel color often determines the attractiveness of an orange to the consumer, the effects of HLB on this important characteristic are of great concern within the fresh fruit citrus industry. Symptomatic oranges from HLB-affected trees (HLB-SY) are greener or less orange in peel color compared to asymptomatic oranges from HLB-affected (HLB-AS) or from HLB-unaffected trees (healthy). Several studies investigated changes in peel color due to infection by CLas. A less orange-colored peel was reported in symptomatic Hamlin fruit (Baldwin et al., 2010, 2018; Liao and Burns, 2012). However, variation in peel color of Valencia oranges depended on harvest date and year (Baldwin et al., 2010, 2018; Liao and Burns, 2012; Massenti et al., 2016) suggesting that Valencia orange may be less prone to peel color changes due to HLB. Valencia fruit has naturally more color than Hamlin and, therefore, HLB effect on peel color would be less visible.

### Sugar and Organic Acids

The physicochemical characteristics of oranges play a vital role in determining the quality of the orange juice produced. There is no general agreement among available results in the scientific literature regarding pH due to CLas infection. The pH of orange juice from HLB-infected trees were either higher, lower, or similar compared to juice made with oranges from uninfected trees (Plotto et al., 2008, 2010; Raithore et al., 2015; Dala Paula et al., 2018).

TA, SSC, and SSC/TA tend to be similar in juice from asymptomatic HLB-AS and healthy oranges. However, a few studies reported differences, although small, in SSC/TA between HLB-AS and healthy Valencia and Hamlin orange juices (Baldwin et al., 2010; Dagulo et al., 2010; Massenti et al., 2016; Hung and Wang, 2018). Juice from HLB-SY fruit usually presents the highest TA, and the lowest SSC and SSC/TA in Valencia, Hamlin (**Tables 2** and **3**), Westin and Pera orange juices (Bassanezi et al., 2009). Recent studies reported variation among fruit affected by the disease, with higher SSC in juice from HLB-SY Hamlin (Baldwin et al., 2018; Hung and Wang, 2018) and Valencia (Baldwin et al., 2018) and a higher SSC/TA in juice from HLB-SY Hamlin compared to juice from healthy fruit (Hung and Wang, 2018). Recently, uninfected trees are difficult to find in Florida, which explains why in the Hung and Wang (2018)

**TABLE 2 |** Physicochemical characteristics of Valencia orange juice made with healthy fruit and fruit at different stages of HLB infection.

| References | Orange juice sample | | Physicochemical characteristics | | | |
|---|---|---|---|---|---|---|
| | Harvest time | Status or conditions | pH | TA (g/100 mL) | SSC (°Brix) | SSC/TA |
| **VALENCIA ORANGE JUICE** | | | | | | |
| Plotto et al., 2008[I] | July 2006 | Healthy FJ | 4.62 b | 0.64 a | 12.0 a | 18.8 b |
| | | HLB FJ | 4.78 a | 0.54 b | 11.3 a | 21.0 b |
| | | Healthy JWP | 4.60 b | 0.63 a | 11.6 a | 18.3 b |
| | | HLB JWP | 4.74 b | 0.47 c | 10.1 b | 21.6 a |
| Bassanezi et al., 2009[II] | Blend of different harvests[1] | HLB-AS | – | 1.22 b | 9.6 a | 8.3 a |
| | | HLB-SY | | 1.75 a | 8.0 b | 4.8 b |
| Baldwin et al., 2010[I] | March 2007 | Healthy | – | 0.82 a | 10.7 a | 13.2 a |
| | | HLB-AS | | 0.84 a | 10.3 a | 12.5 a |
| | April 2007 | Healthy | | 0.68 a | 10.1 a | 15.1 a |
| | | HLB-AS | | 0.72 a | 9.7 a | 13.6 a |
| | May 2007 | Healthy | | 0.57 a | 10.6 a | 18.6 a |
| | | HLB-AS | | 0.54 a | 9.6 b | 18.0 a |
| | June 2007 | Healthy | | 0.43 a | 11.0 a | 25.8 a |
| | | HLB-AS | | 0.41 a | 10.1 b | 24.8 a |
| Dagulo et al., 2010[III] | April 04, 2008 | Healthy | – | – | – | 13.7 a |
| | | HLB-AS | | | | 10.8 b |
| | | HLB-SY | | | | 5.10 c |
| | April 18, 2008 | Healthy | | | | 14.8 a |
| | | HLB-AS | | | | 13.0 b |
| | | HLB-SY | | | | 5.57 c |
| | May 23, 2008 | Healthy | | | | 18.2 b |
| | | HLB-AS | | | | 21.5 a |
| | | HLB-SY | | | | 9.8 c |
| Plotto et al., 2010[IV] | April 2008 | Healthy | 3.78 | 0.89 | 14.5 | 16.2 |
| | | HLB-AS | 3.68 | 1.05 | 14.7 | 14.1 |
| | June 2008 | Healthy | 4.37 | 0.42 | 12.0 | 28.7 |
| | | HLB-AS | 4.27 | 0.46 | 13.2 | 28.4 |
| Liao and Burns, 2012[V] | April 2009 | Healthy | – | 0.85 b | 11.6 a | 13.5 a |
| | | HLB-AS | | 0.85 b | 11.2 a | 13.1 a |
| | | HLB-SY | | 0.91 a | 9.3 b | 10.2 b |
| Slisz et al., 2012[IV] | May 2007 | Healthy | – | 0.54 | 10.6 | 19.5 |
| | | HLB-AS | | 0.52 | 9.6 | 18.5 |
| | June 2007 | Healthy | | 0.40 | 10.8 | 27.3 |
| | | HLB-AS | | 0.38 | 9.7 | 25.7 |
| | | HLB-SY | | 0.69 | 6.9 | 10.1 |
| Raithore et al., 2015[III] | April 2009 | Healthy | 4.17 a | 0.62 b | 12.2 a | 19.7 a |
| | | HLB-SY | 3.81 a | 1.14 a | 11.6 a | 10.2 b |
| Massenti et al., 2016[III] | March + May 2013 | Heatlhy | – | 0.72 b | 12.4 a | 11.0 a |
| | | HLB-AS | | 0.75 b | 12.2 a | 10.4 b |
| | | HLB-SY | | 1.22 a | 8.5 b | 4.5 c |
| Baldwin et al., 2018[IV] | April 2015 | Healthy | 4.20 | 0.68 | 11.6 | 17.1 |
| | | HLB-SY | 4.12 | 0.75 | 11.8 | 15.9 |
| Dala Paula et al., 2018[VI] | March 2013 | Healthy | 4.35 a | 0.72 b | 10.5 a | 14.6 a |
| | | HLB-SY | 3.86 b | 0.94 a | 9.6 b | 10.1 b |

*TA, titratable acidity; SSC, soluble solids content; FJ, filtered juice; JWP, juice with pulp; AS, asymptomatic; SY, symptomatic; Healthy-R, fruit harvest from healthy not shaken trees; Healthy-R, fruit harvest from healthy shaken trees (healthy-retain); Healthy-D, healthy fruit that dropped to the ground upon shaking the trees (healthy-drop); HLB-R, fruit retained on shaken HLB affected-trees; HLB-D, fruit that dropped from HLB affected-trees.*

[1] *Blend of oranges harvested on September 2004, July and October 2005, and August 2007;*

*Values from the same reference with the same letter within columns and same harvest time do not differ in disease status, according to statistical analysis ([I] Fisher's test significant difference test at $p \leq 0.05$; [II] Student t-test with the probability of error estimated to be lower than 0.000; [III] ANOVA and Tukey's test $p \leq 0.05$; [IV] not applicable; [V] Duncan's multiple range test $p \leq 0.01$; [VI] ANOVA and Tukey's test $p \leq 0.05$ for SSC, and $p \leq 0.001$ for TA and SSC/TA).*

**TABLE 3 |** Physicochemical characteristics of Hamlin orange juice made with healthy fruit and fruit at different stages of HLB infection.

| References | Orange juice sample | | Physicochemical characteristics | | | |
|---|---|---|---|---|---|---|
| | Harvest time | Status or conditions | pH | TA (g/100 mL) | SSC (°Brix) | SSC/TA |
| **HAMLIN ORANGE JUICE** | | | | | | |
| Bassanezi et al., 2009[I] | Fruits of different harvests[1] | HLB-AS | – | 0.76 a | 9.6 a | 13.1 a |
| | | HLB-SY | | 0.91 b | 8.9 b | 10.7 b |
| Baldwin et al., 2010[II] | December 2007 | Healthy | – | 0.49 a | 7.8 a | 16.0 a |
| | | HLB-AS | | 0.50 a | 7.6 a | 15.3 a |
| | February 2008 | Healthy | | 0.59 a | 11.6 a | 19.8 b |
| | | HLB-AS | | 0.50 a | 10.4 b | 22.0 a |
| Plotto et al., 2010[III] | February 2008 | Healthy | 4.19 | 0.50 | 11.9 | 23.8 |
| | | HLB-AS | 4.17 | 0.52 | 11.4 | 22.1 |
| Liao and Burns, 2012[IV] | December 2007 | Healthy | – | 0.75 a | 11.3 a | 15.1 a |
| | | HLB-AS | | 0.80 a | 11.5 a | 14.3 ab |
| | | HLB-SY | | 0.78 a | 9.1 b | 11.7 b |
| Raithore et al., 2015[V] | January 2009 | Healthy | 4.22 a | 0.52 a | 11.4 a | 21.7 a |
| | | HLB-SY | 4.22 a | 0.52 a | 11.3 a | 21.7 a |
| Baldwin et al., 2018[III] | December 2014 | Healthy | 4.37 | 0.41 | 9.2 | 22.5 |
| | | HLB-SY | 3.82 | 0.44 | 8.8 | 21.9 |
| | January 2015 | Healthy | 4.37 | 0.42 | 11.2 | 26.7 |
| | | HLB-SY | 4.28 | 0.46 | 11.6 | 25.7 |
| Hung and Wang, 2018[V] | December 2016 + January 2017 | Healthy | – | 0.87 b | 7.4 c | 8.5 c |
| | | HLB-AS | | 0.92 a | 9.9 a | 10.8 a |
| | | HLB-SY | | 0.96 a | 8.9 b | 9.3 b |

*TA, titratable acidity; SSC, solid soluble content; AS, asymptomatic; SY, symptomatic; Healthy-R, fruit harvest from healthy not shaken trees; Healthy-R, fruit harvest from healthy shaken trees (healthy-retain); Healthy-D, healthy fruit that dropped to the ground upon shaking the trees (healthy-drop); HLB-R, fruit retained on shaken HLB affected-trees; HLB-D, fruit that dropped from HLB affected-trees.*

[1] *Blend of oranges harvested on July 2007, June and July 2008.*

*Values from the same reference with the same letter within columns and same harvest time do not differ in disease status, according to statistical analysis ([I] Student t-test with the probability of error estimated to be lower than 0.000; [II] Fisher's test significant difference test at $p \leq 0.05$; [III] not applicable; [IV] Duncan's multiple range test $p \leq 0.01$; [V] ANOVA and Tukey's test $p \leq 0.05$).*

study, Hamlin healthy oranges were from young 2-year old trees grown under protective screens while HLB-SY or HLB-AS oranges were obtained from older field-grown trees, making the comparison not as accurate as if trees were of the same age and growing conditions. SSC/TA, a parameter commonly used as a fruit quality index, tends to increase at later harvest dates and is more heavily affected by harvest time and orange cultivar than HLB infection status (Baldwin et al., 2010). Among the orange cultivars investigated, evaluation of the effects of HLB predominantly addresses Valencia oranges.

Glucose, fructose, and sucrose were quantified in orange juice from HLB-infected trees and compared with juice from oranges from uninfected trees. In the early studies, glucose and fructose either did not vary, or slightly decreased upon the effect of disease status in fruit (Plotto et al., 2008; Baldwin et al., 2010; Slisz et al., 2012; Raithore et al., 2015; **Table 4**). Only recent studies reported a significant increase of glucose and fructose content in juice from HLB-SY fruit compared with healthy oranges (Baldwin et al., 2018; Dala Paula et al., 2018). On the other hand, sucrose and total sugar contents decreased in juice made with oranges from HLB-affected trees in most studies, and more notably, in juices from HLB-SY Valencia and Hamlin oranges. The change in sugars in HLB-SY fruit reflects the disruption in the plant carbohydrate metabolism reported in leaves of citrus affected by HLB (Fan et al., 2010), as well as the impaired sugar transport due to the disease (Liao and Burns, 2012; Chin et al., 2014; Zheng et al., 2018). An increase in cell-wall invertase was observed in HLB-infected leaves resulting in a decrease in sucrose content (Fan et al., 2010). Cell-wall invertase is a glycoprotein enzyme generally found in developing sink organs (roots and fruits) responsible for the hydrolysis of sucrose into glucose and fructose. Asymptomatic (HLB-AS and healthy) oranges can have sucrose contents ∼2.5 times higher than that of symptomatic fruit (Slisz et al., 2012). In addition, Fan et al. (2010) suggested that *C*Las prefers to use fructose causing an accumulation of glucose and sucrose, which are metabolic resources but also signaling components that interfere through feedback inhibition on photosynthesis and contribute to HLB's yellowing leaf mottle symptoms. Poiroux-Gonord et al. (2013) also demonstrated an increase in sucrose content in the pulp of oranges next to leaves submitted to photooxidative stress despite the fact that the studied "Navelate" orange trees were not infected by *C*Las and, consequently, had no blocking or impaired transportation of the phloem sap as one

**TABLE 4 |** Contents of sugars and acids of Valencia and Hamlin orange juice made with healthy fruit and fruit at different stages of HLB infection.

| References | Orange juice sample | | Sugars (g/100 mL) | | | | Organic acids (g/100 mL) | |
|---|---|---|---|---|---|---|---|---|
| | Harvest time | Status or conditions | Glucose | Fructose | Sucrose | Total sugars | Citric acid | Malic acid |
| **VALENCIA ORANGE JUICE** | | | | | | | | |
| Plotto et al., 2008[I] | July 2006 | Healthy FJ | 2.8 a | 1.9 a | 4.3 a | – | 0.52 a | 0.13 a |
| | | HLB FJ | 2.8 a | 1.9 a | 4.1 a | | 0.45 b | 0.10 b |
| | | Healthy JWP | 2.6 ab | 1.8 ab | 4.1 ab | | 0.48 ab | 0.11 b |
| | | HLB JWP | 2.5 b | 1.7 b | 3.7 b | | 0.40 c | 0.09 c |
| Baldwin et al., 2010[I] | March 2007 | Healthy | 1.9 a | 1.9 a | 4.9 a | 8.7 a | – | – |
| | | HLB-AS | 1.9 a | 1.9 a | 4.7 a | 8.6 a | | |
| | April 2007 | Healthy | 1.9 a | 2.0 a | 5.2 a | 9.1 a | | |
| | | HLB-AS | 1.7 a | 1.8 a | 4.4 b | 8.0 b | | |
| | May 2007 | Healthy | 2.0 a | 2.0 a | 5.5 a | 9.5 a | | |
| | | HLB-AS | 1.8 b | 1.9 a | 4.8 b | 8.5 b | | |
| | June 2007 | Healthy | 2.0 a | 2.0 a | 5.6 a | 9.7 a | | |
| | | HLB-AS | 1.8 b | 1.9 a | 4.8 b | 8.4 b | | |
| Liao and Burns, 2012[II] | April 2009 | Healthy | – | – | – | 7.1 a | – | – |
| | | HLB-AS | | | | 6.8 a | | |
| | | HLB-SY | | | | 1.8 b | | |
| Slisz et al., 2012[III] | May 2007 | Healthy | 1.4 a | 1.7 a | 4.1 a | – | 0.64 a | 0.26 a |
| | | HLB-AS | 1.3 a | 1.6 a | 3.7 a | | 0.57 a | 0.23 a |
| | June 2007 | Healthy | 1.4 a | 1.7 a | 4.6 a | | 0.47 a | 0.26 a |
| | | HLB-AS | 1.2 a | 1.6 a | 3.9 a | | 0.38 a | 0.22 a |
| | | HLB-SY | 1.1 a | 1.5 a | 1.5 b** | | 0.91 b* | 0.18 b* |
| Raithore et al., 2015[IV] | April 2009 | Healthy | 2.2 a | 2.3 a | 5.0 a | – | 0.53 b | 0.17 a |
| | | HLB-SY | 2.7 a | 2.7 a | 3.4 b | | 1.40 a | 0.12 b |
| Baldwin et al., 2018[V] | April 2015 | Healthy | 2.0 | 2.2 | 5.4 | 9.6 | 0.74 | 0.20 |
| | | HLB-SY | 2.2 | 2.5 | 5.2 | 9.8 | 0.80 | 0.19 |
| Dala Paula et al., 2018[VI] | March 2013 | Healthy | 2.0 b | 2.3 b | 5.6 a | 10.0 a | 0.84 b | 0.14 a |
| | | HLB-SY | 2.3 a | 2.7 a | 4.2 b | 9.0 b | 1.41 a | 0.11 b |
| **HAMLIN ORANGE JUICE** | | | | | | | | |
| Baldwin et al., 2010[I] | December 2007 | Healthy | 1.5 a | 1.5 a | 3.9 a | 7.0 a | – | – |
| | | HLB-AS | 1.3 b | 1.4 a | 3.2 b | 6.0 b | | |
| | February 2008 | Healthy | 2.2 a | 2.2 a | 5.4 a | 9.8 a | | |
| | | HLB-AS | 1.8 b | 1.8 b | 4.0 b | 7.6 b | | |
| Raithore et al., 2015[IV] | January 2009 | Healthy | 2.9 a | 3.0 a | 5.4 a | – | 0.53 a | 0.16 a |
| | | HLB-SY | 2.7 a | 2.7 a | 4.7 a | | 0.55 a | 0.17 a |
| Baldwin et al., 2018[V] | December 2014 | Healthy | 1.6 | 1.6 | 4.6 | 7.8 | 0.63 | 0.21 |
| | | HLB-SY | 1.7 | 1.8 | 3.8 | 7.3 | 0.65 | 0.23 |
| | January 2015 | Healthy | 2.2 | 2.2 | 5.5 | 9.9 | 0.66 | 0.23 |
| | | HLB-SY | 2.4 | 2.4 | 5.3 | 10.0 | 0.73 | 0.21 |

*FJ, filtered juice; JWP, juice with pulp; AS, asymptomatic; SY, symptomatic; Healthy-R, fruit harvest from healthy not shaken trees; Healthy-R, fruit harvest from healthy shaken trees (healthy-retain); Healthy-D, healthy fruit that dropped to the ground upon shaking the trees (healthy-drop); HLB-R, fruit retained on shaken HLB affected-trees; HLB-D, fruit that dropped from HLB affected-trees.*

*Values from the same reference with the same letter within columns and same harvest time do not differ in disease status, according to statistical analysis ([I]Fisher's test significant difference test at $p \leq 0.05$; [II]Duncan's multiple range test $p \leq 0.01$; [III]p-values represent comparisons within harvest *$p \leq 0.05$; **$p \leq 0.001$; [IV]ANOVA and Tukey's test $p \leq 0.05$; [V]not applicable; [VI]ANOVA and Tukey's test $p \leq 0.05$ for glucose, total sugars and malic acid, and $p \leq 0.01$ for sucrose, fructose and citric acid).*

of the different mechanisms attributable to the CLas (Hijaz et al., 2016).

For organic acids, the majority of the studies reported similar citric and ascorbic acid levels in juice from HLB-unaffected fruit and asymptomatic oranges from HLB-affected trees. However, juice from HLB-SY oranges generally has higher content of citric acid and lower content of malic acid compared to juice from healthy fruit (**Table 4**). Poiroux-Gonord et al. (2013) reported an increase in organic acid, especially succinic acid, in the pulp of oranges nearby leaves submitted to photooxidative stress, a situation associated with HLB effects in citrus leaves (Cen et al., 2017).

## Secondary Metabolites

Oranges are an important source of secondary metabolites which promote human health, particularly flavonoids, limonoids, hydroxycinnamic acids, and polyamines. Many secondary metabolites result from the interaction between the plant and its environment, and are induced by biotic and abiotic factors. Changes in the levels of certain classes of secondary metabolites in oranges are frequently due to stress conditions in plants, including the photooxidative stress in nearby leaves (Poiroux-Gonord et al., 2013). In addition, these compounds are influenced by many factors, such as: cultivar, cultivating methods, degree of ripeness, and processing and storage conditions (Sudha and Ravishankar, 2002; Ramakrishna and Ravishankar, 2011; Chin et al., 2014).

Generally, higher concentrations of phenolic compounds are found in sprouts and seedlings compared to mature plants, consistent with the notion that plant phenolics provide a degree of protection against predation (Drewnowski and Gomez-Carneros, 2000). Similarly, there is an increase of phenolic compounds levels in fruit and leaves from HLB-infected trees (Dagulo et al., 2010; Hijaz et al., 2013a; Kiefl et al., 2018). Flavonoids, particularly hesperidin, narirutin and dydimin, were higher in the peel, pulp and juice of HLB-symptomatic fruit (Massenti et al., 2016; Dala Paula et al., 2018; Kiefl et al., 2018) in comparison with the respective fruit parts from unaffected trees. The pulp of HLB-symptomatic Valencia oranges from two different harvests (March and May 2013) showed an increase of 148 and 17% in narirutin, respectively, and an increase of 86 and 94% in hesperidin, respectively, compared to the corresponding healthy fruit pulp (Massenti et al., 2016). Juice from HLB-SY Valencia oranges harvested in March 2013, contained higher amounts of tangeretin (>4x), nobiletin (>2x), heptamethoxyflavone (>1.5x), diosmin (>2x), didymin (>1.5x), vicenin-2 (>1.5x), nomilin (>20x), limonin (>7.5x), and limonin glucoside (>1.5x) compared to juice from HLB-unaffected oranges (Dala Paula et al., 2018).

In general, juice made with asymptomatic oranges from HLB-infected trees is more similar to juice made with oranges from HLB-unaffected trees when compared to juice made with symptomatic fruit regarding secondary metabolite content. When differences are present, they are caused by harvest maturity rather than by disease status (Baldwin et al., 2010). The interaction of fruit maturity and HLB is not well understood, but Dagulo et al. (2010) suggested that fruit symptomatic for HLB are similar to immature fruit (lower sugars, higher acids, higher bitter limonoids), which is probably why the effect of HLB is more prevalent early in the season. They also suggested that HLB-affect fruit are slow to mature, likely due to a compromised vascular system. Baldwin et al. (2010) determined several secondary metabolites, including hydroxycinnamic acids at 6.3 min and 7.2 min; vicenin-2; feruloyl putrescine; narirutin 4′-glucoside; limonin glucoside; narirutin; nomilin glucoside; nomilinic acid glucoside; limonin and nomilin in asymptomatic and healthy juice made with Hamlin oranges harvested in December 2007. Feruloyl putrescine was the only secondary metabolite that was present at similar levels. However, the same orange cultivar harvested in February 2008 presented similar levels of the two hydroxycinnamic acids; vicenin-2; feruloyl putrescine, limonin glucoside, narirutin, and nomilin glucoside between healthy and asymptomatic juices. The same comparison performed with Valencia oranges harvested in April 2008, had similar contents of all of the secondary metabolites; however, oranges from the June harvest showed different levels of feruloyl putrescine, limonin glucoside, and limonin. These results demonstrate that harvest maturity has greater effect on the content of secondary metabolites than *C*Las infection (Baldwin et al., 2010).

Juice made with HLB-affected oranges contains high levels of nomilin and limonin, more so when made from symptomatic oranges. Both, nomilin and limonin are known to provide bitterness in citrus fruit and its juice (Maier et al., 1977, 1980; Hasegawa et al., 2000). Early research on the effect of HLB on fruit quality suggested that limonin levels >1 mg/L could induce bitterness in juice (Plotto et al., 2010) as it was also the detection threshold in water (Guadagni et al., 1973). However, further research showed that the recognition threshold of limonin was actually around 4–6 mg/L in a complex matrix such as orange juice (Guadagni et al., 1973; Dea et al., 2013). In fact, it is now recognized that only symptomatic oranges have their taste compromised (Baldwin et al., 2010; Plotto et al., 2010; Slisz et al., 2012; Chin et al., 2014; Raithore et al., 2015; Dala Paula et al., 2018) and only severely affected orange juice has limonin levels above 4 mg/L (**Table 5**). This suggests that there are other compounds involved with the bitter taste of juice from symptomatic oranges (Dala Paula et al., 2018), and that interactions of flavonoids together with the combination of lower sugars with higher acids enhances limonoid bitterness perception (Dea et al., 2013; Kiefl et al., 2018).

## Amino Acids and Bioactive Amines

The accumulation of proline, arginine, and branched chain amino acids is expected in plants subjected to conditions that induce stress, such as drought, high salinity and acidity, high incidence of light, high concentration of heavy metals in the soil, changes in temperature, as well as response to biotic stress, such as plant diseases (Rai, 2002; Sharma and Dietz, 2006; Slisz et al., 2012; Malik et al., 2013). Studies showed that proline was higher in leaves of symptomatic HLB-infected trees (Cevallos-Cevallos et al., 2011, 2012; Malik et al., 2014), but it was lower in juice from HLB-SY Valencia fruit (Slisz et al., 2012). In contrast, Hung and Wang (2018) reported an accumulation of proline in Hamlin orange juice from HLB-infected trees. These authors suggested that some of the control trees of the Slisz et al. (2012) study possibly tested as false negatives due to the detection limit of PCR methods or uneven distribution of *C*Las throughout the tree. However, in both studies the amino acids alanine, arginine, leucine, isoleucine, threonine, and valine were found at lower concentrations in juice from HLB-symptomatic oranges (Slisz et al., 2012; Hung and Wang, 2018).

In juice from HLB-symptomatic Valencia and Hamlin oranges, the concentrations of asparagine and phenylalanine were over two times higher than in juice from healthy oranges, and histidine content also increased (Chin et al., 2014). An increase of asparagine and histidine contents was also found in juice from HLB-symptomatic Valencia fruit (Slisz et al., 2012)

**TABLE 5 |** Limonin-glucoside, limonin and nomilin contents of Valencia and Hamlin orange juice made with healthy fruit and fruit at different stages of HLB infection.

| References | Orange juice sample | | Secondary metabolites (mg/L) | | |
|---|---|---|---|---|---|
| | Harvest time | Status or conditions | Limonin-glucoside | Limonin | Nomilin |
| **VALENCIA ORANGE JUICE** | | | | | |
| Baldwin et al., 2010[I] | March 2007 | Healthy | 123.2 a | 0.90 b | 0.22 b |
| | | HLB-AS | 123.4 a | 1.37 a | 0.66 a |
| | April 2007 | Healthy | 122.4 b | 0.78 b | 0.30 b |
| | | HLB-AS | 137.6 a | 1.24 a | 0.54 a |
| | May 2007 | Healthy | 134.9 a | 0.67 b | 0.12 b |
| | | HLB-AS | 137.7 a | 1.40 a | 0.26 a |
| | June 2007 | Healthy | 115.4 b | 0.52 b | 0.06 a |
| | | HLB-AS | 144.4 a | 0.93 a | 0.11 a |
| Slisz et al., 2012[II] | May 2007 | Healthy | 530 b | 3.29 b | – |
| | | HLB-AS | 716 a*** | 4.71 a* | |
| | June 2007 | Healthy | 716 a | 2.82 c | |
| | | HLB-AS | 911 a | 5.18 b** | |
| | | HLB-SY | 976 a | 7.53 a | |
| Raithore et al., 2015[III] | April 2009 | Healthy | – | 0.85 b | 0.22 b |
| | | HLB-SY | | 2.34 a | 0.69 a |
| Kiefl et al., 2018[IV] | February 2015 | Healthy | 240 | 10 | <LOQ*** |
| | | HLB-SY | >250 | 11 | <LOQ |
| | March 2015 | Healthy | 180 | <LOQ** | nd |
| | | HLB-SY | >250 | 8 | <LOQ |
| | April 2015 | Healthy | 220 | <LOQ | nd |
| | | HLB-SY | >250 | <LOQ | nd |
| Baldwin et al., 2018[IV] | April 2015 | Healthy | 147.8 | 1.4 | 0.1 |
| | | HLB-SY | 126.9 | 4.2 | 1.4 |
| Dala Paula et al., 2018[V] | March 2013 | Healthy | 48.3 b | 1.2 b | 0.1 b |
| | | HLB-SY | 92.0 a | 9.3 a | 1.1 a |
| **HAMLIN ORANGE JUICE** | | | | | |
| Baldwin et al., 2010[I] | December 2007 | Healthy | 72.3 b | 1.45 b | 0.43 b |
| | | HLB-AS | 102.0 a | 3.27 a | 0.83 a |
| | February 2008 | Healthy | 132.1 a | 0.82 b | 0.18 b |
| | | HLB-AS | 141.7 a | 1.54 a | 0.51 a |
| Raithore et al., 2015[III] | January 2009 | Healthy | – | 0.64 b | 0.06 b |
| | | HLB-SY | | 2.44 a | 0.25 a |
| Kiefl et al., 2018[IV]* | November 2014 | Healthy | 110 | 8.3 | 9.7 |
| | | HLB-SY | >250 | 16 | 11 |
| | January 2015 | Healthy | 140 | <LOQ | <LOQ |
| | | HLB-SY | >250 | 13 | <LOQ |
| Baldwin et al., 2018[IV] | December 2014 | Healthy | 33.7 | 1.3 | 0.1 |
| | | HLB-SY | 34.9 | 2.2 | 0.3 |
| | January 2015 | Healthy | 47.2 | 0.8 | 0.1 |
| | | HLB-SY | 56.1 | 1.1 | 0.2 |

*<LOQ, below limit of quantification; nd, not detectable.*

*Values from the same reference with the same letter within columns and same harvest time do not differ in disease status, according to statistical analysis ([I]Fisher's test significant difference test at $p \leq 0.05$ for limonin glucoside and $p \leq 0.01$ for limonin and nomilin; [II]p-values represent comparisons within harvest *$p \leq 0.05$; **$p \leq 0.001$; [III]ANOVA and Tukey's test $p \leq 0.05$; [IV]not applicable; [V]ANOVA and Tukey's test $p \leq 0.001$).*

**The results were converted from mg/Kg to mg/L assuming orange juice's density of 1.0 g/cm$^3$; **LOQ of limonin = 1.2 mg/Kg; ***LOQ of nomilin = 5.0 mg/Kg.*

and in Satsuma orange leaves (Malik et al., 2014). A suggested explanation for this trend is that *C*Las may have inhibited the tree defense mechanism which, in turn, reduced the action of proline dehydrogenase, an enzyme responsible for the activation of the biosynthetic pathways of proline from ornithine and glutamate. Thus, the levels of this amino acid could not increase (Slisz et al., 2012). However, the accumulation of phenylalanine in juice from HLB-affected oranges (Slisz et al., 2012) differs

from results from Malik et al. (2014) and Hung and Wang (2018). These last authors explained that phenylalanine is an essential precursor for secondary phenylpropanoid metabolism by phenylalanine ammonialyase in higher plants and its gene expression is significantly affected by *C*Las infection (Hung and Wang, 2018).

Hamlin and Valencia HLB-symptomatic oranges showed high contents of the aromatic amine synephrine, however, juice from HLB-asymptomatic and healthy fruit had similar content (Slisz et al., 2012; Chin et al., 2014). In plants, putrescine is a necessary diamine precursor of polyamines synthesis (spermidine and spermine), and its increase is usually associated with environmental stress (Coelho et al., 2005; Gloria, 2006; Sharma and Dietz, 2006); however, putrescine content was not affected in juice from HLB-symptomatic oranges (Chin et al., 2014). On the other hand, feruloyl putrescine, a conjugate of putrescine and ferulic acid, is found at high concentrations in juice from HLB-symptomatic Hamlin oranges compared to juice from HLB-asymptomatic and healthy fruit. The same trend does not seem to be observed in Valencia oranges (Baldwin et al., 2010).

## Effect of HLB on the Levels and Profile of Volatile Compounds

The orange flavor has been studied more than any other citrus flavor. Unlike grapefruit, lemon, and lime, in which there are one or two flavor-impact compounds, the orange flavor is the result of a combination of volatiles in specific proportions. Among the various components that contribute to the distinct flavor of the orange, the most important are: terpenes (d-limonene, myrcene, α-pinene, valencene); aldehydes (acetaldehyde, *E*-2-pentenal, hexanal, octanal, nonanal, decanal, sinensal, neral, and geranial, the last two sometimes called citral); esters (ethyl acetate, ethyl propionate, methyl butanoate, ethyl butanoate, ethyl 2-methylpropanonate, ethyl 2-methylbutanoate, ethyl 3-hydroxyhexanoate); alcohols (ethanol, *E*-2-hexen-1-ol, *Z*-3-hexen-1-ol, linalool, α-terpineol); and ketones (1-octen-3-one, β-damascenone, β-ionone) (Shaw, 1991; Perez-Cacho and Rouseff, 2008).

Only a few studies have dealt with changes in the volatile compounds in orange juice affected by HLB (Baldwin et al., 2010; Dagulo et al., 2010; Hung and Wang, 2018; Kiefl et al., 2018). These studies have shown that monoterpenes tend to be higher and esters lower in juice affected by HLB (Baldwin et al., 2010; Dagulo et al., 2010; Kiefl et al., 2018). These studies have also shown that sesquiterpenes, including valencene, were typically lower in HLB-affected juice (**Figure 3**). These results are relevant to the quality of orange juice as esters typically impart fruity flavor and terpenes are characteristic of citrus volatiles: ethyl acetate, ethyl butanoate and ethyl hexanoate have sweet fruity odors in orange juice (Plotto et al., 2008). Ethyl-3-hydroxyhexanoate is reported as one of the major esters in orange juice (Shaw, 1991; Fan et al., 2009) with a sweet and fruity odor (Buettner and Schieberle, 2001). Lower esters and higher terpenes are likely to result in imbalanced flavor of orange juice. While the terpene alcohol linalool, with a fruity/floral characteristic, is desired in orange juice, other terpene alcohols (α-terpineol, 4-terpineol, carveol) are indicators of oxidation and poor quality (Dagulo et al., 2010; Kiefl et al., 2018). Dagulo et al. (2010) suggested that the higher terpenes and lower sesquiterpenes in HLB-affected orange juice might be an indication of lower enzyme activity in the pathway converting terpenes to sesquiterpenes of the affected oranges.

Contradictory results were reported for alcohols. Dagulo et al. (2010) and Baldwin et al. (2010) found that (*Z*)-3-hexenol was higher in juice from HLB-affected Valencia oranges, while Kiefl et al. (2018) found it was higher in juice from healthy fruit. In fact, Dagulo et al. (2010) and Hung and Wang (2018) found that all alcohols were higher in HLB-affected juice. The levels of aldehydes varied much more depending on the study, season and cultivar. Octanal, nonanal, and decanal are important aldehydes with a characteristic citrus odor (Perez-Cacho and Rouseff, 2008) and were higher in juice from "healthy" oranges in the Kiefl et al. (2018) and Baldwin et al. (2010) studies. On the contrary, these aldehydes were higher in juice from HLB-asymptomatic Valencia oranges in the Dagulo et al. (2010) study. Likewise, the "green" odor compound hexanal was 65 to 110% higher in samples from HLB-unaffected samples in the Baldwin et al. (2010) study, up to 81% higher in HLB-symptomatic Valencia in the Dagulo et al. (2010) study and about 25% higher in HLB-affected fruit (Kiefl et al., 2018). Considering all three studies, it is important to remember that volatile levels differ with harvest times, types of processes used to prepare the orange juice (Baldwin et al., 2012b) and HLB infection status. It is important to emphasize that, generally, asymptomatic orange juice is similar to healthy orange juice with respect to volatile profile.

Not only does HLB affect the profile of volatiles in orange juice, but by having an effect on fruit size, peel oil extracts are reduced by 30% in HLB-symptomatic fruit (Bai et al., 2017). As in orange juice, sesquiterpene hydrocarbons are lower in the peel oil of symptomatic fruit, as are some monoterpenes and straight-chain aldehydes. In another study, Xu et al. (2017) found compounds only detected in oil from HLB-affected fruit, including β-longifolene and perillene, two terpenes, and 4-decenal, an aldehyde. However, these authors admit that more samples should be analyzed to confirm these findings. These authors found that linalool, decanal, citronellol, citral, carvone, and dodecanal were higher in the oil from asymptomatic than symptomatic fruit from Hamlin and Valencia oranges harvested twice in the season (Xu et al., 2017). Kiefl et al. (2018) analyzed peel oil by gas chromatography and olfactometry and found that mostly odor-active aldehydes contributed to the difference between healthy and HLB-affected Valencia oil, being higher in HLB-affected fruit.

Emission of volatiles from orange tree is believed to play an important role in the plant—vector APC interaction. Orange leaves emit almost all juice volatiles except esters (Hijaz et al., 2013b, 2016). ACP infestation stimulated 21 out of the 27 volatiles by 2- to 10-fold in orange leaves, in comparison with *C*Las-infection which only stimulated four volatiles—d-limonene, β-phelandrene, citronellal, and undecanal by 4- to 20- fold (Hijaz et al., 2013b). In another experiment, Hijaz et al. (2016) showed that HLB tolerant cultivars contained higher amounts

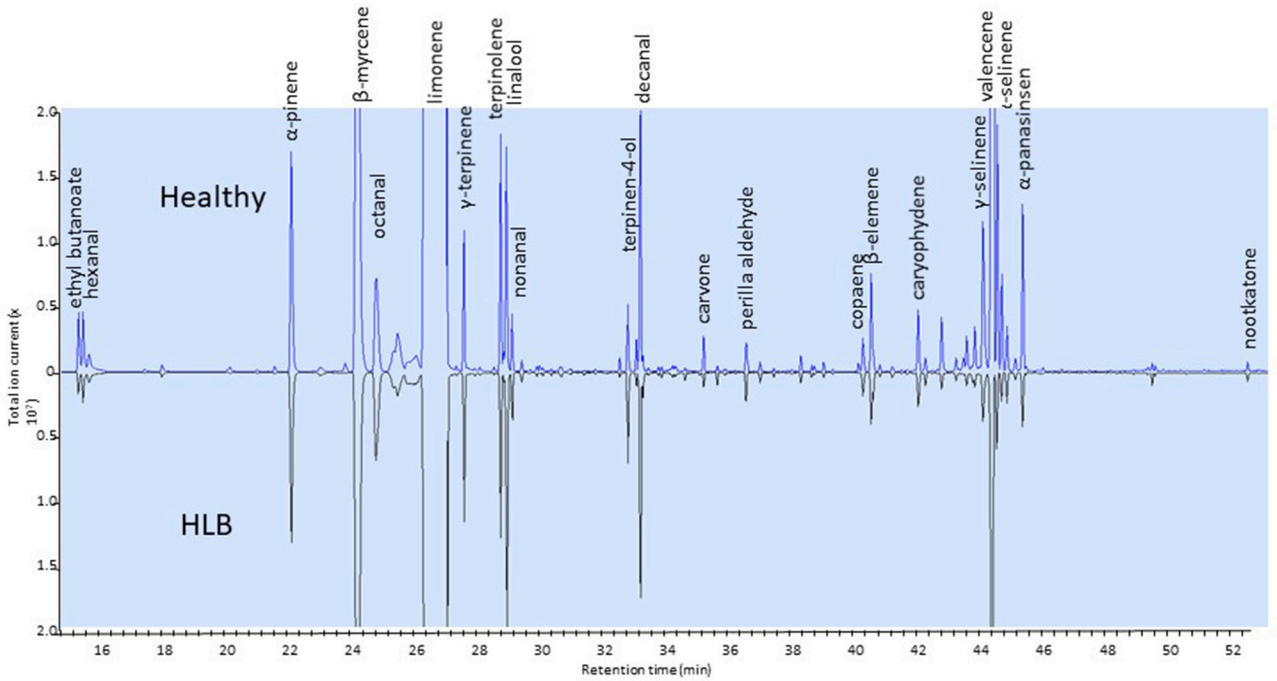


**FIGURE 3 |** Side-by-side chromatograms of headspace volatiles of juice samples extracted from healthy **(top)** and HLB **(bottom)** Valencia oranges. Ethyl butanoate (ester) and sesquiterpenes are in greater amount in healthy than in HLB juice.

of volatiles, especially those showing antimicrobial activities, including aldehydes (undecanal, neral, geranial, and citronellal), and mono/sesquiterpene hydrocarbons and derives (linalool, d-limonene, myrcene, α- and β- phellandrene, E-caryophellene, β- and γ-elemene, germacrene D, and geranyl acetate).

## Effects of HLB on Juice Sensory Characteristics

Early reports describing the symptoms of HLB disease on trees, leaves, and citrus fruit were published in plant pathology journals, and effects on fruit were mostly describing the visual defects. One report mentioned HLB-symptomatic oranges as having a "bitter and salty taste, especially in the early part of the season" (McClean and Schwarz, 1970). These were informal observations about fruit having off flavor. Only recently formal sensory analyses (triangle test, difference-from-control test) have been used to describe and quantify other, and more subtle taste attributes in HLB-affected fruit (Plotto et al., 2008). Studies have included analysis of juice prepared from fruit of healthy, unaffected trees, and of juice prepared from asymptomatic and symptomatic fruit from HLB-affected trees testing positive for *C*Las. Comparisons were made using difference-from-control tests where panelists rated the degree of difference between healthy and infected juice. Sensory results could be explained by chemical data and confirmed differences between healthy, asymptomatic, and symptomatic fruit/juice. These comparisons were repeated with several cultivars, Hamlin, Mid-Sweet and Valencia, and the differences between healthy and HLB-affected fruit were more pronounced and obvious to the palate with fruit harvested early than late in the season (Baldwin et al., 2010; Plotto et al., 2010). Juice made with these symptomatic, HLB-affected oranges had the most off-flavors, commonly described as "bitter," "sour," and "sour/fermented." Higher bitterness and sourness in symptomatic fruit could be explained by higher levels of limonin and titratable acidity and with lower soluble solids content (Baldwin et al., 2010). A trained panel provided more insight into the various descriptors characterizing orange juice made with HLB-symptomatic fruit, with several negative descriptors regarding taste and flavor (astringency, tingling, harshness, bitterness, metallic-taste, low sweetness, saltiness/umami, musty, sourness/fermented, pungent/peppery, low citrusy taste; **Tables 6**, **7**), usually due to an imbalance in the chemical composition in the affected fruit (Baldwin et al., 2010, 2012a, 2018; Plotto et al., 2010, 2017; Raithore et al., 2015; Dala Paula et al., 2018; Kiefl et al., 2018).

HLB off-flavor in severely symptomatic fruit is so pronounced that processing healthy with affected fruit is likely to negatively impact the sensory quality of commercial orange juice (Bassanezi et al., 2009). Juice from HLB-symptomatic fruit, up to 25%, can be blended with juice from unaffected fruit without being perceived as off-flavored for both Hamlin and Valencia (Raithore et al., 2015). Another study found an even lower amount (10% by juice mass) of HLB-symptomatic fruit being acceptable in a blend (Ikpechukwu, 2012). Both studies were performed with not-from-concentrate juice processed in a pilot plant, and can be a basis to processors who need to sort symptomatic fruit out before juicing to maintain overall juice quality (Raithore et al., 2015). No studies were found with juice made from concentrate,

**TABLE 6 |** Sensorial descriptors ascribed to Huanglongbing in Valencia orange juice.

| Sensorial descriptor* | Harvest time | Juice specifications | References# |
|---|---|---|---|
| Acidic | July 2006, April 2008 | Frozen juice with pulp and filtered[I], hand-squeezed juice [II] | 1; 4 |
| Astringent | June 2008; March 2013; April 2015 | Commercially processed juice[III]; premium setting[IV] | 2; 5; 6 |
| Bitter/slight bitter | June 2008; March 2013 | Commercially processed juice | 2; 6 |
| Bland | June 2008 | Commercially processed juice | 2 |
| Burning | March 2013; April 2015 | Premium setting, commercially processed juice | 5; 6 |
| Fermented | July 2006 | Frozen juice with pulp and filtered | 1 |
| Grapefruit-like flavor | April 2008, 2015; June 2008; March 2013; | Commercially processed juice, premium setting | 2; 5; 6 |
| Green flavor | March 2013; April 2015 | Premium setting, commercially processed juice | 5; 6 |
| HLB-bitter*** | Monthly basis during the season 2014 and 2015 | Hand-squeezed juice | 4 |
| Less body** | April 2015 | Premium setting | 5 |
| Less fruity non-citrus flavor** | March 2013; April 2015 | Premium setting, commercially processed juice | 5; 6 |
| Less orange flavor** | March 2013; April 2015 | Premium setting, commercially processed juice | 5; 6 |
| Less sweet** | April 2008, 2015; March 2013 | Commercially processed juice, premium setting | 2; 5; 6 |
| Metallic | June 2008; April 2009; 2015 | Commercially processed juice | 3; 5 |
| Off flavor | April 2008 | Commercially processed juice | 2 |
| Overripe | July 2006 | Frozen juice with pulp and filtered | 1 |
| Oxidized oil | April 2015 | Premium setting | 5 |
| Peel oil | April 2008, 2015; June 2008; March 2013; | Commercially processed juice, premium setting | 2; 5; 6 |
| Salty/umami | April 2009; March 2013 | Commercially processed juice | 3; 6 |
| Sharp | April 2008, June 2008 | Commercially processed juice | 2 |
| Sour | April 2008; 2009; 2015; March 2013; | Commercially processed juice, premium setting | 2; 3; 5; 6 |
| Stale | March 2013 | Commercially processed juice | 6 |
| Sweeter** | April 2008 | Commercially processed juice | 2 |
| Tangy | April 2008 | Commercially processed juice | 2 |
| Tingly | April 2009; 2015 | Commercially processed juice, premium setting | 3; 5 |
| Typical HLB flavor | March 2013; April 2015 | Premium setting, commercially processed juice | 5; 6 |
| Umami | April 2015 | Premium setting | 5 |
| Unidentifiable different flavor | June 2008 | Commercially processed juice | 2 |
| Weak in taste | July 2006 | Frozen juice with pulp and filtered | 1 |

**The list of sensorial descriptors includes commentaries realized by the panel during sensory evaluations and attributes significantly higher in asymptomatic or symptomatic orange juice, CLas (+), compared to healthy juice (control).*

***In comparison with healthy orange juice (control), CLas (–).*

****According to the authors, HLB-bitter refers to a long-lasting metallic, astringent and harsh taste.*

*[I] Frozen juice thawed overnight served with the pulp and without pulp. Juice was filtered then flash pasteurized at 71°C for 10 s and immediately cooled then served;*

*[II] Oranges were hand juiced and lightly pasteurized using at 71°C for 15 s, and frozen at −20°C;*

*[III] Oranges were extracted using a commercial JBT 391 single head extractor with premium juice extractor settings and pasteurized under simulated commercial conditions (1.2 L/m, 8 to 10 s hold time, 83 to 90°C).*

*[IV] Oranges were extracted as is a customary industry practice, premium setting was selected according to the particular characteristic of the peel oil specific to Valencia, it was passed through a pressure filtration finisher with screen size 0.51 mm and then pasteurized under simulated commercial conditions (1.2 L/m, 90°C).*

*#References: [1] Plotto et al. (2008); [2] Plotto et al. (2010); [3] Raithore et al. (2015); [4] Kiefl et al. (2017); [5] Baldwin et al. (2018); [6] Dala Paula et al. (2018).*

but processors always blend those juices and add volatiles which can mask some off-flavors.

More in-depth studies on bitterness in orange juice revealed that the two known bitter limonoids in orange juice, limonin, and nomilin, act in a synergistic manner and their thresholds of perception are lower when tasted together (Dea et al., 2013). Furthermore, both limonoids have a different taste characteristic: limonin is described as "bitter" whereas nomilin is described as "metallic" by some panelists, probably contributing to the taste synergy. Unlike other tastes, the detection thresholds for bitter molecules are generally extremely low, and can have prolonged aftertaste. Perception of bitterness is highly variable among humans, and because there are more than 50 known bitter receptors, studies of bitterness associated with juice affected by HLB are complex. Fractionated liquid chromatography of orange juice combined with taste analysis revealed that derivative molecules of hydroxycinnamic acids had bitter and astringent taste, and were more prevalent in juice from HLB-symptomatic oranges (Dala Paula et al., 2018). Using the same technique, Glabasnia et al. (2018) identified 10 polymethoxylated flavones (PMFs) that enhanced bitterness due to limonin and nomilin in orange juice. Tasted without limonin and nomilin in a model solution, these PMFs increased astringency but not bitterness. These studies demonstrate the complexity of interactions between molecules belonging to two chemical classes—polyphenols and limonoids, on taste

**TABLE 7 |** Sensorial descriptors ascribed to Huanglongbing in Hamlin orange juice.

| Sensorial descriptor* | Harvest time | Juice specifications | References# |
|---|---|---|---|
| Astringent | February 2008; December 2014; January 2015 | Commercially processed juice[I], standard industry setting[II] | 1; 4 |
| Bitter | December 2007; 2014; February 2008; January 2009; 2015 | Hand-squeezed juice[III], commercially processed juice, standard industry setting | 1; 2; 4 |
| Burning | December 2014; January 2015 | Standard industry setting | 4 |
| Cooked | January 2009 | Commercially processed juice | 2 |
| Earthy | February 2008 | Commercially processed juice | 1 |
| Fatty | February 2008 | Commercially processed juice | 1 |
| Fermented | February 2008 | Commercially processed juice | 1 |
| Grapefruit-like | December 2007; 2014; February 2008; January 2009; 2015 | Hand-squeezed juice, commercially processed juice, standard industry setting | 1; 2; 4 |
| Green flavor | December 2014; January 2015 | Standard industry setting | 4 |
| HLB bitter*** | Monthly basis during the season 2014 and 2015 | Hand-squeezed juice | 3 |
| Less body** | December 2014 | Standard industry setting | 4 |
| Less freshness** | February 2008 | Commercially processed juice | 1 |
| Less fruity non-citrus flavor** | December 2014 | Commercially processed juice | 4 |
| Less orange flavor** | February 2008 | Commercially processed juice | 1 |
| Less sweet** | February 2008; December 2014; January 2015 | Commercially processed juice, standard industry setting | 1, 4 |
| Metallic | February 2008; December 2014; January 2015 | Commercially processed juice, standard industry setting | 1; 4 |
| Musty | February 2008 | Commercially processed juice | 1 |
| Overripe | January 2009 | Commercially processed juice | 2 |
| Oxidized oil | December 2014; January 2015 | Standard industry setting | 4 |
| Peel oil/citrus oil | December 2007; 2014; February 2008; January 2009; 2015 | Hand-squeezed juice, commercially processed juice, standard industry setting | 1; 2; 4 |
| Peppery | February 2008 | Commercially processed juice | 1 |
| Pungent | February 2008 | Commercially processed juice | 1 |
| Salty/umami | February 2008 | Commercially processed juice | 1 |
| Sharp | December 2007 | Hand-squeezed juice | 1 |
| Sour | December 2007; 2014; February 2008; January 2009; 2015 | Hand-squeezed juice, commercially processed juice, standard industry setting | 1; 2; 4 |
| Stale | December 2014; January 2015 | Standard industry setting | 4 |
| Sour milk | December 2007 | Hand-squeezed juice | 1 |
| Sulfury | January 2009 | Commercially processed juice | 2 |
| Tingly | February 2008; December 2014; January 2015 | Commercially processed juice, standard industry setting | 1; 4 |
| Typical HLB flavor | December 2014; January 2015 | Standard industry setting | 4 |
| Umami | December 2015; January 2015 | Standard industry setting | 4 |

**The list of sensorial descriptors includes commentaries realized by the panel during sensory evaluations and attributes significantly higher in asymptomatic or symptomatic orange juice, CLas (+), compared to healthy juice (control);*

***In comparison with control juice—healthy orange juice, CLas (–).*

****According to the authors, HLB-bitter refers to a long-lasting metallic, astringent and harsh taste.*

*[I]Oranges were extracted using a commercial JBT 291 single head extractor with premium juice extractor settings and pasteurized under simulated commercial conditions (1.2 L/m, 8 to 10 s hold time, 82 to 90°C).*

*[II]Oranges were extracted as is a customary industry practice, premium setting was selected according to the particular characteristic of the peel oil specific to Hamlin, it was passed through a pressure filtration finisher with screen size 0.51 mm and then pasteurized under simulated commercial conditions (1.2 L/m, 90°C).*

*[III]Oranges were hand juiced and lightly pasteurized using at 71°C for 15 s, and frozen at −20°C;*

*#References: [1]Plotto et al. (2010); [2]Raithore et al. (2015); [3]Kiefl et al. (2017); [4]Baldwin et al. (2018).*

perception. Contribution of volatiles, sugars, acids, amino acids, and high molecular weight carbohydrates such as pectin to flavor and taste adds to the complexity of understanding the effect of HLB on juice quality.

A new technology was developed to predict HLB-affected orange juice quality by measuring pathogen *C*Las titer using real-time PCR (Bai et al., 2013; Zhao et al., 2018). Fruit severely infected by HLB may have one or more of the following juice quality features: low sugar, abundant bitter limonoids, and rich acid/sourness, but the common feature for all juice prepared from such fruit is high *C*Las titer, which correlated negatively with sensory characteristics (Bai et al., 2013; Zhao et al., 2018). The U.S. patent by Zhao et al. (2018) is the only study where *C*Las is quantified in orange juice from many sources showing an attempt of quantifying the degree of infection. The amount of *C*Las titer in the juice (lower CT values) negatively correlated with sweetness,

orange and fruity flavor, and positively with negative attributes, such as off flavor and "umami."

## FINAL CONSIDERATIONS

HLB affects the sensory and physicochemical characteristics of orange juice despite the available scientific literature data which presents contradictory results among these parameters. This may be due to factors such as: different harvest times of the oranges, differences in the age of the trees between the control group and HLB group, unpredictable environmental stress, as well as the level of *C*Las infection of the orange trees. Juice made with HLB-symptomatic fruit usually has high TA, low SSC and SSC/TA, whereas juice made with asymptomatic fruit from HLB-infected trees is generally similar to juice processed with healthy fruit. In general, HLB causes a decrease in sucrose, total sugars and malic acid contents while ascorbic acid does not seem to be significantly affected by the disease. On the other hand, levels of citric acid, bitter limonoids (limonin and nomilin), hydroxycinnamic acids, flavonoids (particularly tangeretin), nobiletin, narirutin, hesperidin, diosmin, and didymin are higher in juice from HLB-symptomatic oranges compared to juice from healthy fruit. The content of amino acids, alanine, arginine, asparagine, histidine, isoleucine, leucine, phenylalanine, proline, threonine, and valine are altered by HLB. Additionally, symptomatic Hamlin orange juice has high synephrine and feruloyl putrescine levels.

Regarding the typical HLB-off flavor in orange juice, the loss of sweetness can generally be explained by lower sucrose and total sugar levels and SSC, along with higher citric acid, and sourness is explained by higher TA and citric acid content. Furthermore, some volatiles may contribute to increased or decreased perception of sweetness or sourness (Bartoshuk et al., 2017; Plotto et al., 2017). Elevated levels of limonin and nomilin are partially responsible for the typical HLB-bitterness. These two limonoids have a synergistic effect which decreases their perception and identification thresholds in orange juice. Beyond these compounds, there is evidence indicating that other compounds, possibly hydroxycinnamic acids, are involved with the typical HLB-bitterness (Dea et al., 2013; Dala Paula et al., 2018). Unquestionably, more work is needed to further identify the full list of compounds contributing to the unpleasant taste and mouthfeel in HLB-affected orange juice. Sensory studies take into consideration that the lower sugar contents reinforce the perception of bitterness.

There are relatively few published papers evaluating the effects of HLB on orange juice's chemical, physicochemical and, especially, sensorial qualities and most of the research available was performed using Valencia oranges, followed by Hamlin. While citrus fruit sold as fresh can be substantially devalued by loss of color and misshape, juice processors still can process oranges that are HLB-symptomatic as long as they are mixed with asymptomatic fruit in <25% ratio of HLB-SY to asymptomatic fruit (healthy or HLB-AS). Processors traditionally add back flavor extracts from orange peel oil or orange essence to standardize juice (Ringblom, 2004), and have that tool to modulate citrus flavor and sweetness. Other attempts have been made to isolate compounds, or groups of compounds from citrus juice, peel or molasses, which could also increase sweetness or decrease bitterness perception in HLB-affected orange juice (Kiefl et al., 2017). More research to mitigate HLB-induced off-flavors and tastes could include use of resins, that are already used to remove bitter limonoids; the proper resin that only removes bitter compounds without removing flavor volatiles would need to be designed. Also tailoring aroma packages to mask bitterness or enhance sweetness, or adding non-volatiles extracted from oranges that mask bitterness. Finally, perhaps adding substances that bind bitter limonoids in the juice and then remove, or adding enzymes that glycosylate bitter limonoids, rendering them non-bitter. These efforts are likely to be pursued until a long-term solution is found to citrus greening disease.

## AUTHOR CONTRIBUTIONS

BD-P, AP, JB, JM, EB, RF, and MG contributed to the writing and review of the manuscript.

## ACKNOWLEDGMENTS

We thank CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), Capes (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for providing scholarship and Fapemig (Fundação de Amparo a Pesquisa do estado de Minas Gerais) for financial support.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01976/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor is currently co-organizing a Research Topic with one of the authors RF, and confirms the absence of any other collaboration.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 30 January 2019
doi: 10.3389/fpls.2019.00041

# Implications of Heat Treatment and Systemic Delivery of Foliar-Applied Oxytetracycline on Citrus Physiological Management and Therapy Delivery

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OPEN ACCESS

**Edited by:**
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**Reviewed by:**
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**Specialty section:**
*This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science*

**Received:** *28 September 2018*
**Accepted:** *11 January 2019*
**Published:** *30 January 2019*

**Citation:**
*Vincent C, Pierre M, Li J and Wang N (2019) Implications of Heat Treatment and Systemic Delivery of Foliar-Applied Oxytetracycline on Citrus Physiological Management and Therapy Delivery. Front. Plant Sci. 10:41. doi: 10.3389/fpls.2019.00041*

Huanglongbing is an economically devastating disease of citrus in Florida and around the world. This study was undertaken to assess two grower-used therapies, heat treatment, and foliar anti-bacterial application. Specifically, there was an industry claim that heat treatment improved subsequent systemic uptake of foliar-applied anti-bacterial compounds. We hypothesized that new vegetative growth induced by heat treatment could lead to increased foliar delivery because of a greater number of new leaves in which cuticles would be more permeable. The study included two factors (1) heat treatment (with or without) and (2) pruning, in which all new leaves, all mature leaves, or no leaves were removed. A commercial formulation of oxytetracycline (OTC) was applied to plants with a non-ionic penetrant surfactant, but one branch on each tree was covered to assess direct versus systemic delivery. The study was repeated twice, destructively assessing whole-plant leaf area and dry weights, as well as OTC content in directly applied and covered leaves. Heat treatment and defoliation treatments reduced growth, but did not affect systemic delivery of OTC. OTC was detected in nearly all covered leaf samples in both repetitions, though at lower concentrations than in directly applied leaves. We conclude that neither heat treatment nor leaf age strongly affect systemic OTC delivery. Implications of this study for leaf age effects on foliar delivery and for phloem delivery of foreign compounds through foliar application are discussed.

**Keywords: citrus, huanglongbing (HLB), anti-microbial delivery, heat treatment, xenobiotic delivery**

## INTRODUCTION

Huanglongbing disease ("citrus greening"; HLB) is one of the most economically damaging diseases of perennial crop plants. It is caused by the persistent infection of a phloem-limited bacterium *Candidatus* Liberibacter asiaticus (Las), which leads to hyperaccumulation of carbohydrates in leaves, and starvation of sink tissues (Cimò et al., 2013), resulting in reduced overall growth, leaf senescence, loss of root length and mass density, reduced fruit production and increased pre-harvest fruit drop (Achor et al., 2010; Gottwald et al., 2014; Johnson et al., 2014;

Wang et al., 2017). The disease has no cure, and no resistant citrus germplasm has been found. By 2014, 9 years after its detection in Florida, United States, HLB was estimated to have reduced the statewide sweet orange crop by 100 million boxes per year, approximately 4 million metric tons (Spreen et al., 2014).

Because of the economic impacts of HLB, therapies to reduce populations of Las within infected plants in the field have been an area of research as well as grower practice. These therapies include steam or hot-water generated heat treatments and the use of anti-bacterial compounds (Doud et al., 2014; Hu et al., 2017).

Heat treatment, sometimes called "thermotherapy," has been effective in eliminating Las from citrus without killing the citrus, when applied under controlled conditions (Hoffman et al., 2012). In field studies, Las titer has been reduced but not eliminated with 48 h treatments of 42°C or 1 min treatments of 60°C without killing the plant (Doud et al., 2014; Jia et al., 2017), though assessments of treatments on other plant variables such as photosynthesis were not considered in these studies. In response to these positive results in terms of reducing bacterial titer, some companies have begun to market heat-treatment services in commercial citrus groves.

Although a range of compounds have been assessed for efficacy against Las (Yang et al., 2016), currently there are two active ingredients that are approved for use in citrus under an emergency exemption (EPA, Section 18c) to address the HLB crisis affecting Florida citrus. These are streptomycin and oxytetracycline hydroxide (OTC). Large scale in-field trials of streptomycin and OTC treatments are ongoing (B. Shatters, USDA-ARS, *personal communication*; S. Slinski, Citrus Research and Development Foundation, *personal communication*). Several studies have indicated that OTC and, to a lesser degree, streptomycin can dramatically reduce Las titer when injected into the trunk (Hu and Wang, 2016; Hu et al., 2017), although this application method is not approved for agricultural use. Because these products are new to use in citrus, the degree to which they can be delivered into leaves and the degree to which they become systemic in the plant have not been shown.

The current study responded to claims that heat treatment increased the uptake of foliar-applied anti-bacterial compounds (S. Slinski, Citrus Research and Development Foundation, *personal communication*). Because we observed heat treatments to induce a new flush of vegetative growth, we hypothesized that the new flush could improve anti-microbial uptake. This was based on the observation that young leaves have thinner cuticles and that there are differences in cross-cuticular agrochemical delivery among leaf ages (Orbović et al., 2001). This study was designed to test the effect of treatments on citrus plants, not to test the efficacy of treatments against Las. Although previous work had addressed the movement of OTC after trunk injection, no study had assessed systemic delivery of commercial anti-bacterial compounds after foliar application, which is the only on-label method of application (Hu and Wang, 2016; Hu et al., 2017). Thus we designed and implemented a study to test: (1) the effect of heat treatment on citrus growth and systemic OTC delivery, (2) whether leaf age mediated the heat treatment effect, and (3) the degree of systemic OTC delivery from foliar application.

# MATERIALS AND METHODS

The same study and treatment design were repeated twice: July 6, 2017–November 17, 2017 (Trial 1) and September 20, 2017–January 26, 2018 (Trial 2).

## Plant Materials and Experimental Setup

Thirty-six 'Valencia'/X-639 trees at 1 year past budding from a local nursery were repotted in 30-gallon (113 L) plastic containers in sandy soil taken from the field. Three days later, trees were placed in the ground with the top of the container at ground level in the field, in Lake Alfred, FL, United States (28.1021° N, 81.7121° W). The pots allowed us to isolate the entire root system for destructive sampling at the end of the study. Submerging the pots in the soil allowed us to imitate typical field conditions, preventing heating the rootzone any more than would occur in a field setting. Thus we reproduced a typical field heat treatment, while maintaining the ability to assess the entire root system of each tree. Each plant was irrigated 1 h daily with a 4 L $h^{-1}$ drip emitter. A commercial slow-release fertilizer (14-3-11 N-P-K) was applied to the soil surface around each tree at a rate of 17 g of total nitrogen per tree.

## Experimental Design

The experiment was set out as a randomized complete block design with six blocks. The treatment structure was a factorial design with heat treatment and pruning factors. Heat treatment had two levels: (1) with heat treatment (Heat) and (2) without heat treatment (No Heat). Pruning had three levels: (1) old leaves removed with only young leaves remaining (Young Leaves), (2) young leaves removed with only mature leaves remaining (Mature Leaves), and (3) no leaves removed with all leaves remaining (All Leaves). Blocks were arranged in a row of pots. Plants were assigned randomly to block. The experimental unit was an individual plant in a single pot.

## Treatments

Twenty-five days after repotting and placing trees in the ground, 18 trees were treated with steam-generated heat. The first repetition heat treatment was imposed by a local heat treatment company (Premier Energy, Inc., Woodstock, GA, United States). The parameters of the first repetition heat treatment were heat between 43 and 54°C with the high temperature held for no longer than 45 s. The second repetition heat treatment was imposed by University of Florida heat treatment personnel. Actual temperatures for the second repetition are shown in **Figure 1**. Generally these temperatures were higher for longer than those of the first repetition. In both repetitions, heat treatment consisted of a tent placed on each treated tree for 60–180 s. Immediately after heat treatment, each tree was tipped to induce new flush. Tipping consisted of pruning every shoot tip on the plant, removing the tip but no leaves. One day after tipping, one branch in each tree was covered and tied with plastic bags to prevent direct contact with the foliar application. Where possible, the branch represented approximately 1/4 of the canopy, selecting branches that emerged from below the lowest foliage. Soil was covered with plastic sheeting during application

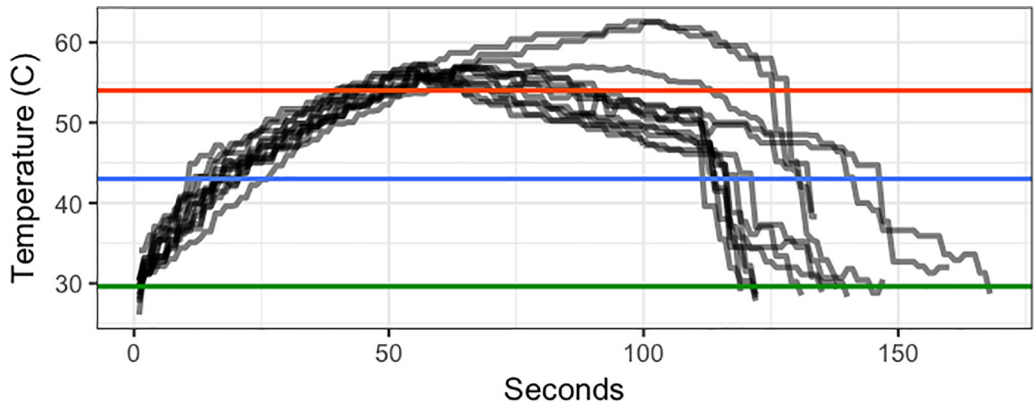


**FIGURE 1 |** Temperature (°C) of heat treatments over time (seconds after start of heating) in Trial 2. Green line shows the ambient temperature. Blue line shows the minimum threshold target temperature (43°C). Red line shows the maximum target temperature (54°C).

to prevent root uptake of OTC. OTC was applied to the entire canopy except branches shielded with plastic bags at a rate of 1 g $L^{-1}$ of OTC (Fireline 17 WP, Agrosource, Inc., Cranford, NJ, United States), with 3.1 ml $L^{-1}$ of Induce non-ionic surfactant (Helena Ag, Collier, TN, United States). Three hundred mL of solution per plant were applied at approximately 400 kPa, which resulted a fine mist and was sufficient to produce runoff from the leaves. One hour later, when trees were completely dried, bags were taken away. Twenty-two days after applying OTC, leaves were collected for OTC quantification. Leaves that received the direct application and those that were covered were sampled separately to assess the difference between contact and systemic delivery. Leaf samples were ground with liquid nitrogen and stored in a −20°F freezer until analysis.

## Variables Measured

Leaf gas exchange (photosynthesis, stomatal conductance, and respiration) and chlorophyll fluorescence variables were measured before heat treatment and 3 weeks after heat treatment. Leaf gas exchange was measured in young fully-mature, sun-exposed leaves. Photosynthesis was measured with in-chamber conditions at 1000 μmol PAR $m^{-2}$ $s^{-1}$, 400 ppm $CO_2$, and 1.5 mPa vapor pressure deficit, using a portable infrared gas analyzer (Li-Cor 6800, Lincoln, NE, United States). Leaf respiration was measured in the pre-dawn dark on the same leaves. Data were recorded in the same leaf of each tree using the same system and parameters, except that the light was turned off.

For OTC quantification samples of three leaves each were collected from the portion of the canopy that received the application and from the bagged portion. Although measurable superficial residue of OTC was unlikely at 22 days after application because of photodegradation (Doi and Stoskopf, 2000), samples were rinsed for 10 s under running distilled water. Subsequently, leaves were frozen in liquid N and ground with liquid N with mortar and pestle, and approximately 600 mg samples were used for analysis. OTC was quantified by HPLC according to Hu and Wang (2016), which describes the efficiencies of OTC recovery and quantification in citrus samples. In addition to OTC concentrations, ratios of concentrations between the directly-applied and bagged samples for each tree were calculated to assess the relative degree of systemic delivery.

At the end of the experiment, leaf areas were measured. Pots were removed from the soil and roots were washed of all soil. Leaves, stems, and fine and structural roots of each tree were individually dried at 60°C for 72 h and weighed. Plant growth variables were calculated as total plant leaf area (LA), average area per leaf (mean LA), and dry weights (DW) of leaves (total leaf DW per plant), stems, fine roots, and structural roots. Total DW was calculated as the sum of all DWs, and shoot:root ratio was calculated as above-ground DWs/total root DWs.

## Data Analysis

For all variables data were analyzed for each Trial separately using the lm command in base R Stats 3.2, using a mixed model with treatments as fixed effects and block as a random effect (R Core Team, 2017). In the case of OTC concentration, subsample (direct application vs. protected leaves) was included as a fixed effect nested within plant (split-plot). For significant effects ($P < 0.05$), means separations were performed using Bonferroni's protected least significant differences, using the agricolae package in R (Mendiburu, 2015).

# RESULTS

## Gas Exchange

Heat treatments induced a noticeable defoliation, as is presented in the plant growth section, and one tree in the Heat-Mature Leaves treatment died in each trial. However, by the time gas exchange and chlorophyll fluorescence were measured 3 weeks later, there were no significant effects on chlorophyll fluorescence, photosynthesis, respiration, or stomatal conductance of the leaves that remained (data not shown due to non-significance of effects).

**TABLE 1 |** *P*-values of plant dry-weight variables at the end of each of two trials of heat treatment and defoliation.

| Trial | Variable | Effect | | |
|---|---|---|---|---|
| | | Pruning | Heat | Pruning × heat |
| 1 | Leaf number | 0.213 | **0.013** | 0.083 |
| | Leaf area | 0.052 | **0.015** | **0.001** |
| | Mean leaf area | **0.036** | 0.073 | 0.053 |
| | Leaf dry weight | 0.054 | **0.008** | **0.002** |
| | Stem dry weight | 0.549 | 0.126 | 0.058 |
| | Structural root dry weight | 0.384 | **0.004** | 0.054 |
| | Fine root dry weight | **0.046** | **0.015** | **0.010** |
| | Total dry weight | 0.169 | **0.007** | **0.006** |
| | Shoot:root ratio | 0.321 | 0.062 | 0.290 |
| 2 | Leaf number | 0.309 | **0.048** | 0.319 |
| | Leaf area | 0.571 | 0.198 | 0.940 |
| | Mean leaf area | 0.739 | 0.911 | 0.415 |
| | Leaf dry weight | 0.426 | 0.132 | 0.943 |
| | Stem dry weight | 0.869 | **0.008** | 0.576 |
| | Structural root dry weight | 0.569 | **0.001** | 0.337 |
| | Fine root dry weight | 0.707 | **0.033** | 0.567 |
| | Total dry weight | 0.633 | **0.003** | 0.822 |
| | Shoot:root ratio | 0.633 | **0.003** | 0.822 |

*Each trial consisted of n = 6 for all combinations and was arranged as a randomized complete block. Values less than P = 0.05 are in bold for clarity.*

## Plant Growth

In Trial 1 there were interactions of pruning and heat treatment for LA, mean LA, leaf DW, structural root DW, fine root DW, and total DW (*P*-values of all effects found **Table 1**). Leaf number was affected by heat only. For all variables, Heat reduced DW or LA accumulation (**Figure 2**, only variables with significant effects are shown). Within the Heat plants Mature Leaves negatively affected growth, while in No Heat plants, Young Leaves negatively affected DW or LA. Fine roots were more affected than structural roots, but the trends among treatments were the same for both variables.

In Trial 2, leaf number, stem DW, structural root DW, fine root DW, total DW, and shoot:root ratio were affected by heat treatment, while no interactions of pruning and heat treatment were found (**Table 1**). In all cases Heat reduced DW relative to No Heat, and increased shoot:root, by affecting roots to a greater degree than shoots (**Figure 3**, only variables with significant effects are shown).

## Oxytetracycline Content

There was no interaction or main effect of heat or pruning on foliar OTC content (see **Table 2** for analyses of variance). However, OTC was detected in nearly all samples of leaves that did not receive direct application, while the effect of covered versus exposed leaves was significant ($P < 0.001$ in both trials; **Table 3**). The proportion of OTC content in covered leaves relative to the content in treatment-exposed leaves varied between the two trials. Although a direct statistical comparison between the two trials is not valid, we note that the 95% confidence intervals did not overlap.

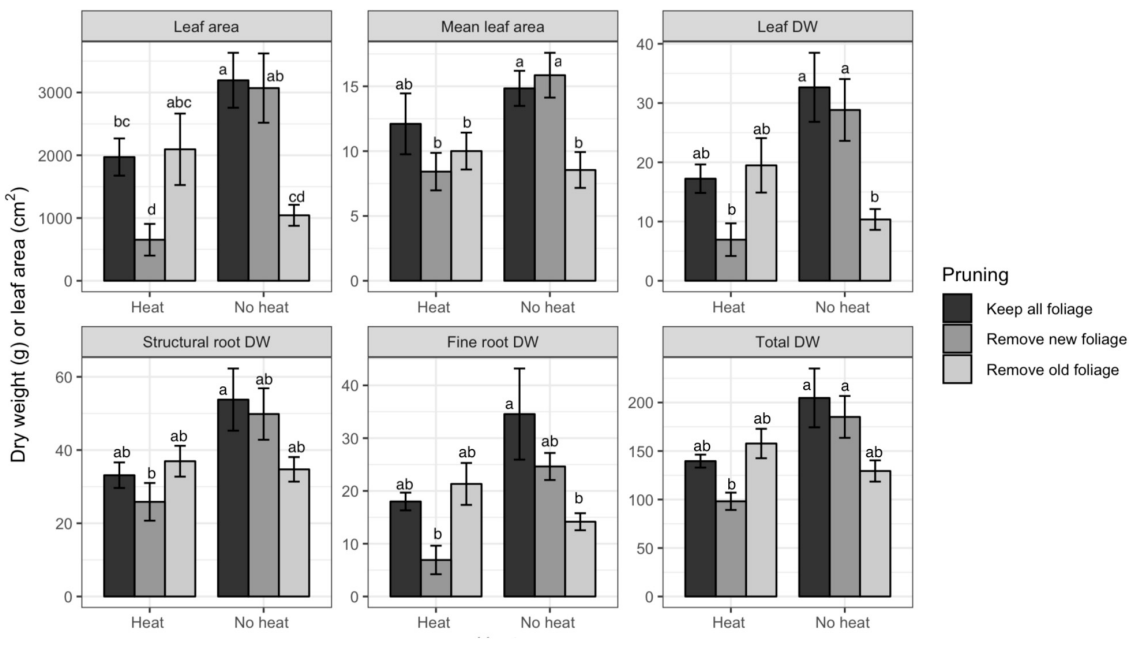


**FIGURE 2 |** Dry weights (DW) and leaf area of various organs of Valencia/X-639 citrus plants upon termination of Trial 1, 81 days after heat treatment. Shaded bars represent means, and error bars represent standard error ($n = 6$). Bars labeled with different letters indicate significant differences at $P < 0.05$ using Bonferroni's protected LSD.

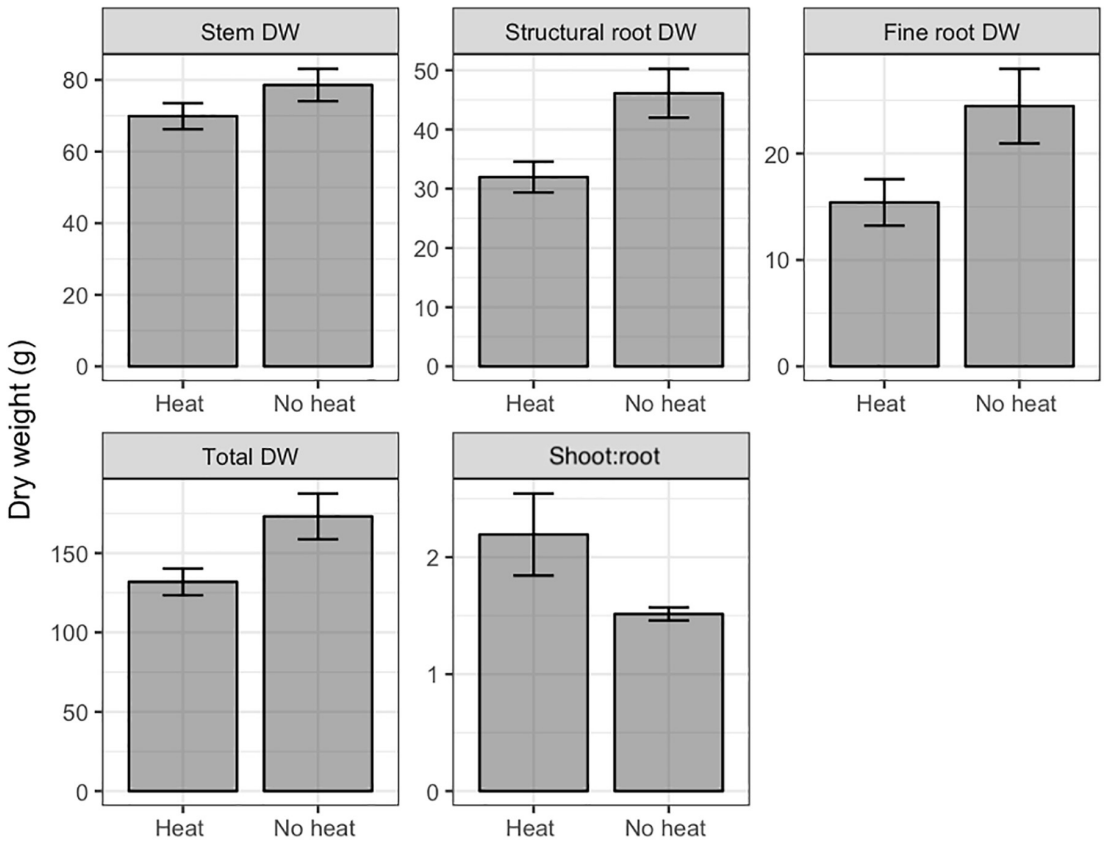


**FIGURE 3 |** Dry weights (DW) and leaf area of various organs of Valencia/X-639 citrus plants upon termination of Trial 2, 111 days after heat treatment. Shaded bars represent means, and error bars represent standard error ($n = 6$). All contrasts significant at $P < 0.05$ using Bonferroni's protected LSD.

# DISCUSSION

## Effects on Growth

Heat treatment negatively affected growth of all plant organs. In Trial 1 the interaction of heat with pruning for most growth variables may be indicative of the defoliation effect, in which many of the mature leaves senesced. This defoliation reduced the total photosynthetic capacity of the tree by reducing photosynthetic area, similar to that found in defoliation treatments by Eissenstat and Duncan (1992). This is reflected in the fact that the Heat-Young Leaves treatment had the same leaf area as Heat-All Leaves, whereas, No Heat-Young Leaves had only about 30% of the total leaf area of the other No Heat pruning treatments (**Figure 2**). Because LA is the primary indicator of photosynthetic capacity, all organ DWs had the same trend as LA (**Figure 2**). This co-occurrence of reduced DW with reduced LA did not carry over to the Trial 2 because treatments did not affect total leaf area. This may be because the trees in Trial 2 had lesser leaf area, and during a low temperature period many leaves senesced in all treatments. An important characteristic of the Heat-induced decrease in DW is that root DW decreased to a greater degree than the other DW variables (**Figure 3**). The prevalent conditions of primarily HLB-infected trees in Florida make root health a priority for production, because HLB has been found to dramatically reduce fine roots and thereby to limit the trees' capacity for water uptake (Kadyampakeni et al., 2014).

## Implications for Foliar Delivery Past the Cuticle

Citrus vegetative growth occurs in spurts called flushes. In Florida, there are usually three vegetative flushes per year. Many growers consider the ideal time of application to be when flush is young (3–4 weeks old; J. Duggar, Peace River Packing, Inc., *personal communication*), based on the observation that the citrus leaf continues to deposit cuticular waxes over its entire lifespan (Syvertsen, 1982). The present results do not support that conclusion for OTC.

Reinforcing this view, studies of foliar delivery of other solutions have been more complex than a linear age-related decrease in delivery. Orbović et al. (2007) found that an adjuvant that reduced surface tension increased Cu delivery into both abaxial leaf surfaces and fruits of grapefruit (*Citrus* × *paradisi*) as compared with adaxial surfaces, indicating that the most likely path of delivery is through stomata. A previous study by the same group found that urea penetration of grapefruit cuticles decreased between 3–4 and 6–7 week leaf age ranges, but that it increased as leaves aged beyond the 6–7 week range (Orbović et al., 2001). In that study, although cuticular wax deposition

continued, much of the cuticle became disorganized, allowing many potential paths of entry of water solutions, indicating that as leaves age, non-stomatal paths of delivery increase.

This conclusion is supported by water permeance studies. Baker et al. (1975) found that citrus leaf cuticular waxes varied greatly in composition, but Geyer and Schönherr (1990) using sour orange (*Citrus aurantium*) found that a wide range of environmental conditions affected composition but did not affect permeance. They concluded that cuticular structure had a greater effect on permeance than thickness or composition within the range of citrus cuticles. Bondada et al. (2006) similarly concluded that cuticular thickness did not govern urea penetration of the cuticle of grapefruit, because, although older leaves on the same stem had thicker cuticles, urea penetration was greater in nodes 1 and 4 than 2 and 3, respectively in basipetal order. Additionally, micrographs of Orbović et al. (2001) indicate stomatal occlusion but also cuticular disruption, possibly diminishing the role of stomata and increasing the role of cuticular organization as leaves age. Thus, the present study is consistent with the body of literature, that mature and young leaves may be equally permeable to foliar-applied products, and we now add that heat treatment is unlikely to alter subsequent delivery.

**TABLE 2 |** Analysis of variance of foliar oxy-tetracycline content in Valencia/X-639 after foliar application at 1 g ai. per L sampled 22 days after application.

| Trial | Effect | Degrees of freedom | *F*-value | *P*-value |
|---|---|---|---|---|
| 1 | Pruning[z] | 2 | 0.55 | 0.54 |
| | Heat[y] | 1 | 1.75 | 0.14 |
| | Subsample[x] | 1 | 24.55 | < 0.0001 |
| | Pruning × heat | 2 | 0.34 | 0.71 |
| | Pruning × subsample | 2 | 0.44 | 0.65 |
| | Heat × subsample | 2 | 0.01 | 0.94 |
| | Heat × pruning × subsample | 4 | 0.56 | 0.58 |
| 2 | Pruning | 2 | 1.24 | 0.31 |
| | Heat | 1 | 2.72 | 0.11 |
| | Subsample | 1 | 295.59 | < 0.0001 |
| | Pruning × heat | 2 | 0.66 | 0.53 |
| | Pruning × subsample | 4 | 0.92 | 0.42 |
| | Heat × subsample | 2 | 0.28 | 0.60 |
| | Heat × pruning × subsample | 4 | 0.19 | 0.83 |

*[z]Pruning consisted of removal of all new leaves, removal of all mature leaves or no leaf removal. [y]Heat consisted of heating above 43°C for greater than 45 s. [x]Subsample contrasts leaves that received direct application with those that were shielded during application.*

**TABLE 3 |** Oxytetracycline (OTC) concentration in leaves which received direct application or were covered during application.

| Trial | Sample | OTC content (μg OTC $g^{-1}$ FW; Mean ± SE) | Relative concentration covered leaves/uncovered leaves (95% CI) |
|---|---|---|---|
| 1 | Applied | 0.53 ± 0.06 a[z] | |
| | Covered | 0.21 ± 0.03 b | 0.36–0.63 |
| 2 | Applied | 0.46 ± 0.07 a | |
| | Covered | 0.14 ± 0.03 b | 0.27–0.34 |

*Samples were collected 22 days after application. [z]Means (n = 36) followed by different letters are significantly different within each trial using Bonferroni's protected LSD (P < 0.05).*

## Implications for Systemic Delivery

Although few studies have addressed systemic antibiotic delivery in citrus directly, a number of studies have addressed the use of anti-microbial compounds against Las. Yang et al. (2016) compared grafting, root drench, and bark paint (at different concentrations for each method) application methods of applying several anti-microbial compounds, and concluded that bark paint at high concentrations (5–6x of commercial label concentrations) was more effective than root drench at lower concentrations. They also found that bark paints of ampicillin and actidione + validoxylamine A significantly reduced Las titer. A subsequent study found that adjuvants that improved ampicillin entry into leaves also significantly reduced Las titer under greenhouse conditions (Yang et al., 2015). Although these studies addressed delivery methods, none considered whether systemic translocation within the plant was achieved after the compound was delivered into the plant. Hu and Wang (2016) addressed translocation dynamics of trunk-injected OTC. They found that trunk injection resulted in OTC detection in leaves in all quadrants of canopy and in roots by 1-week after injection, even with a single port. These treatments greatly reduced Las titer in foliage by 4 days after injection and in roots by 14 days post-injection. A subsequent study indicated that injection of streptomycin also reduced Las titer, but to a lesser degree than OTC, though the effect of combining the two increased efficacy but was not strictly additive (Hu et al., 2017). The limitation of this study, as of others, is that it does not assess phloem-specific delivery of OTC. Phloem delivery is the objective of antibiotic application in HLB management because Las is phloem limited.

## Foliar Delivery for Control of Las

Hu and Wang (2016) applied 2 g of OTC per tree, equivalent to 2.6 foliar applications at the labeled rate of 115 g ai. $ac^{-1}$ with 150 trees per acre. In that study, foliar OTC concentrations reached 0.6–1.5 μg $g^{-1}$ with similar concentrations in roots, as compared to 0.14–0.21 μg $g^{-1}$ in the present study in leaves that did not receive direct application (**Table 2**). In that study there was a reduction of Las titer by 4 days in foliage and by 14 days in roots, indicating that this range of concentrations was sufficient to reduce Las populations. The subsequent study compared injection rates of 1.25 and 2.5 g OTC per plant (Hu et al., 2017), with 60 and 110% reductions in Las titer respectively. Given that the 1.25 to 2.5 g injection per plant range appears to affect efficacy against Las, the lower equivalent rate of foliar application may have a much more muted effect on bacterial titers, even if a high rate of delivery into the leaf is assumed. For purposes of comparison, the recommended rate for foliar application is 1.5 lbs (681 g) per acre of product (AgroSource, Inc., Cranford, NJ, United States), or 125 g OTC per acre. At a standard planting density of 200 trees per acre would result in 0.62 g per tree in the foliar application, with lower quantities

reaching the leaf apoplast, and still lower quantities entering systemic distribution. The low concentrations of OTC in tissues from the present study appear to be sufficiently below those of the trunk injection studies that they may not represent effective levels of Las control.

## CONCLUSION

Heat treatment had negative effects on citrus growth, and did not affect OTC uptake. Leaf age within the grouped ranges assessed in this study also did not affect systemic OTC uptake. However, OTC was delivered into the leaf and was translocated systemically. Future research should address whether tissue concentrations achieved by foliar application are sufficient to reduce Las populations, and whether the pattern of is the same between OTC and streptomycin as the two compounds registered for management of Las in citrus.

## AUTHOR CONTRIBUTIONS

CV and NW conceived the study. MP and CV implemented the study and assessed growth and physiological variables. JL assessed the OTC content. CV, MP, and JL wrote the manuscript. NW revised the manuscript.

## FUNDING

This study was funded by Citrus Research and Development Foundation project number 17-005C.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 31 January 2019
doi: 10.3389/fpls.2018.01890

# Field Evaluation of Integrated Management for Mitigating Citrus Huanglongbing in Florida

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Citrus huanglongbing (HLB) is extremely difficult to control because the psyllid-transmitted bacterial pathogen resides inside the citrus phloem and the disease is systemic. In Florida, the nine billion dollar citrus industry has been significantly impacted by severe HLB epidemics. To combat citrus HLB, in this study we implemented an integrated strategy that includes chemotherapy, thermotherapy, and additional nutrition treatment in three different field trials over three consecutive years. In these trials, only trees already showing HLB symptoms with Ct values ranging from 25.1 to 27.7 were selected for treatments. To assess the complex interactions, we used several methods for evaluating the effectiveness of integrated management, including the slopes (b) of the Ct increase (dy/dt), the pathogenic index (PI) and the decline index (DI) from Ct value and tree scores, and the therapeutic efficacies from PI and DI. This comprehensive analysis showed that most of the tested chemicals were effective to some degree in killing or suppressing the Las bacterium, with higher therapeutic efficacies seen for Grove B, where citrus trees were severely affected by HLB, and it had a higher number of psyllids, relative to Grove E and P in the first 2 years. Trunk-injected penicillin G potassium was the most effective chemical treatment in all groves, followed by Oxytetracycline Calcium Complex, and Silver Nitrate delivered as foliar sprays. Although the steam heat treatment and additional nutrition did not eliminate or suppress Las over the long term, these treatments did positively affect tree growth and recovery in the short term. Overall, our results provide new insights into HLB control method and strategy for integrated management for HLB epidemic plantations.

**Keywords: citrus huanglongbing, integrated management, chemotherapy, thermotherapy, *Candidatus* Liberibacter asiaticus, Las bacterial titers, HLB index**

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***Edited by:***
*Rhuanito Soranz Ferrarezi, University of Florida, United States*

***Reviewed by:***
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***Specialty section:***
*This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Plant Science*

***Received:*** *06 September 2018*
***Accepted:*** *06 December 2018*
***Published:*** *31 January 2019*

***Citation:***
*Zhang M, Yang C, Powell CA, Avery PB, Wang J, Huang Y and Duan Y (2019) Field Evaluation of Integrated Management for Mitigating Citrus Huanglongbing in Florida. Front. Plant Sci. 9:1890. doi: 10.3389/fpls.2018.01890*

## INTRODUCTION

Citrus huanglongbing (HLB) is currently the most devastating citrus disease worldwide. In the state of Florida, millions of infected plants have entered a severe stage of decline with premature fruit drop and dieback. HLB in the United States is associated with *Candidatus* Liberibacter asiaticus (Las), which is transmitted by the Asian citrus psyllid *Diaphorina citri*. Since first documentation of citrus HLB in Florida in 2005, citrus production had declined from 170 million boxes to fewer than 80 million boxes in 2015–2016 (Neupane et al., 2016)

due to HLB in addition to adverse environmental conditions. The production in 2017–2018 was an even smaller harvest of 45 million boxes (https://www.nass.usda.gov/Publications/Todays_Reports/reports/crop0518.pdf).

Furthermore, citrus producers in Florida face a different situation from those in China and Brazil, where relocating citrus to new areas is an effective strategy to establish new plantings of HLB-free citrus. Florida citrus growers have no choice other than to produce citrus on their currently available acreage, regardless of the presence of HLB. However, the impact of HLB on the Florida citrus industry might be mitigated in at least two ways: (1) reducing the HLB pathogen titers and disease index in currently infected trees in existing groves; and (2) preventing and/or curing new infections in new plantings. Thus, management strategies to kill or suppress the HLB bacterium and restore plant vigor are critical. Based on the complex nature of HLB pathosystem and the shortfalls associated with currently available measures in the field, we implemented an integrated therapeutic approach to mitigate HLB effects by reducing Las titers and disease index in field trial.

This integrated therapeutic approach was developed based on previous accomplishments for HLB control that includes development of a chemical screening system to identify effective chemicals for HLB control (Zhang et al., 2010, 2012). More than 120 chemical compounds were evaluated using the optimized graft-based assay to determine activity against Las bacterium and phytotoxicity (Zhang et al., 2010, 2011, 2012, 2014). Of these, 11 have been advanced into these field trials. Because of the systemic nature of HLB infection and the residency of Las in the phloem of infected trees, and the cuticle wax of citrus leaves, oil-in-water and water-in-oil nano-emulsion for application to the bark and as foliar sprays were developed to increase delivery efficiency, respectively (Yang et al., 2015, 2016a).

In addition to chemotherapy, we demonstrated that heat treatment could eliminate Las under controlled conditions (40–42°C for a minimum of 48 h) (Hoffman et al., 2013). Field trials of this approach showed partial success in portable greenhouses that rely on solar energy (Doud et al., 2017). Problems associated with the implementation of thermotherapy in the field include the need to develop techniques that allow the desired temperatures to be attained and maintained within a designated timeframe in a large-scale setting, as well as the need for methods that allow simultaneous treatment of the roots and canopy of infected trees. In previous study, combination therapies that use both heat and chemicals are effective for mitigating HLB infections in greenhouse (Yang et al., 2016b). Here we described the results of a 3-year field trial conducted in the Indian River to examine the therapeutic efficacy of approaches to mitigate HLB that involved an integrated strategy of chemical treatments coupled with heat treatment and additional nutrition.

# MATERIALS AND METHODS

## Field Trial

A 3-year field trial was carried out on a randomized split-plot design to combine chemical, heat and additional nutrition treatments in an overlapping experiment. The 12 chemical treatments were delivered as the major block in each location (grove), whereas two heat treatments was delivered in a sub-split plot, and two additional nutrition was given to a sub-sub-split plot. The data were analyzed as a generalized linear mixed model using the SAS procedure GLIMMIX 8.1. The whole block and split plot factors were treated as fixed effects, and the replication and its interaction with the whole-plot factor were treated as random effects. Differences among treatments were determined with the LINES option of LSMEANS.

These treatments were applied to 192 HLB-positive red grapefruit/sour orange rootstock trees in each of three commercial groves [Groves B (high HLB infection rate), E (moderate HLB infection rate) and P (moderate HLB infection rate)] located in the Indian River. The trees were aged between 3 and 4 years, and the heights ranged from 4 to 8 feet. Additional nutritional and heat treatments were applied to 96 trees in each grove, respectively.

## Chemotherapy

Twelve chemical treatments (**Table 1**) were applied to 16 tree replicates in each grove. All chemicals were applied via foliar spray. In our previous study, trunk application of penicillin was high efficacy against Las (Yang et al., 2016b). Therefore, in this paper, penicillin was given via trunk injection as a positive control.

## Thermotherapy

Heat treatment was given using mobile steam equipment to inject the steam under a portable tent on June 18th, 2015 (grove B) and June 22nd, 2015 (grove E and P). The temperature of the air around the tented tree was raised to 51.6–53.3°C for a period of 30 s. Temperatures were monitored with a sensor placed in each tree.

## Additional Nutrition

In the three groves, Renew® 3-18-20 (Plant Food Systems, Inc., Zellwood, FL) and Citrus Mix (Miller Chemical & Fertilizer, LLC, Hanover, PA) were delivered as foliar sprays at rates of 1 gallon (1 gallon = 3.78541 L) and 1 pound (1 pound = 0.45359 kg) per acre (1 acre = 0.40469 hectare), respectively. Although the chelated micronutrient mix (Citrus Mix) is generally not phytotoxic on its own, its application in combination with Renew® resulted in excessive leaf drop. Therefore, Citrus Mix was replaced by R-TRx liquid micronutrient mix (Diamond R Fertilizer Company, Ft Pierce, FL) and applied at 1 gallon per acre beginning with the second round of treatments, and Renew® 3-18-20 also was applied at 1 gallon. No leaf drop was observed following application of this treatment. In order to provide sufficient nutrition to HLB-affected citrus, the nutritional therapy treatments were applied in addition to the grower/cooperator fertilizer programs, which generally followed UF/IFAS recommendations. Trial Grove B received 3 soil applications of a complete, dry fertilizer mix each year. Trial Grove E received 2 soil applications of a complete slow-release dry mix and monthly applications of N, P, K and micronutrients through the microsprinkler irrigation system each year. Grove P received three soil applications of a complete, dry soluble

**TABLE 1 |** Type and concentration of chemicals applied to citrus trees in the field.

| Chemicals | Code | Concentration and application additives |
|---|---|---|
| Mycoshield® Oxytetracycline Calcium Complex | OXY | 200 ppm with Tactic® surfactant |
| Firewall® Streptomycin Sulfate | Strep | 11 ounces per acre with Tactic® surfactant |
| Aliette® WDG Aluminum tris (O-ethyl phosphonate) | ALI | Five pounds per acre with Kinetic® surfactant |
| Silver Phosphite | SP | Two quarts per acre and a proprietary SP trunk drench formulation applied at 1 liter per tree with Kinetic® surfactant |
| Silver Nitrate | SN | 200 ppm concentration with nano-cre emulsion |
| Carvacrol | CARV | 500 ppm with Makon 12 emulsion |
| P-Cymene | PCY | 500 ppm with Makon 12 emulsion |
| Validoxyamine | VA | 200 ppm with nano-cre emulsion |
| Zhongshengmycin | ZS | 200 ppm with nano-cre emulsion |
| Sulfamethoxine sodium | SDX | 100 ppm with nano-cre emulsion |
| Penicillin G potassium (Positive control) | PEN | 500 ml 5,000 ppm solution, applied by trunk injection 3 times at approximately 1 week intervals |
| Untreated control | CK | |

fertilizer mix and monthly N, P, K, and micronutrients through the microsprinkler irrigation system each year.

The first foliar nutritional treatment application contained Renew® 3-18-20 (urea, dipotassium polyphosphate, phosphorus acid, salicylic acid) at a 1 gallon per acre rate and 1 pound Science Citrus Mix EDTA-chelated micronutrient mix, combined in a tank mix and applied as a foliar spray. Despite of the chelate being an excellent product, when combined with the Renew product, it caused excessive leaf drop. Subsequent treatments throughout the trial contained 1 gallon per acre of RTRx liquid micronutrient mix (zinc, manganese, copper, iron, boron, molybdenum, sulfur and biostimulants as a glucoheptonate complex) instead of the Science Citrus Mix EDTA chelate. The new nutritional tank mix used in the subsequent treatments did not cause any leaf drop.

All chemical treatments were applied in addition to the grower/cooperator spray programs, which did include regular pesticide for psyllid control and foliar micronutrient products, but excluded any antimicrobial treatments. Foliar spray treatments were applied each growing season during the spring (three sprays), summer (one spray) and fall (three sprays) growth flushes (total of seven sprays per year). The first application was applied when the new growth flush first appeared and subsequent applications were made at 14 day intervals so that all new growth would be quickly covered with at least one application. Foliar applications were made with a 100 gallon tank sprayer equipped with a 50 psi (1 psi = 0.06895 bar) handgun calibrated to deliver approximately 150 gallons per acre. Each tree received about 1.0 gallons of finished spray that provided thorough coverage for each tree selected for the trial. Spray treatments were mixed in 25 gallon batches, except for the nutritional treatments that were mixed in 100 gallon batches to spray 96 trees. To avoid residual material on harvested fruit, the last spray of chemical and nutritional treatments delivered by foliar application began on September 25 and ended on October 10, 2015.

Penicillin injection treatments were carried out by drilling two injection sites 2 inches (1 inch = 2.54 cm) deep in the trunk of the treated trees and inserting 7/32 inches (=0.56 cm) injection needles attached by hoses to a plastic bag hung above the injection points, which allowed the solution to enter the tree via gravity feed. New injection sites were drilled in the penicillin (PEN)-treated trees for each time of application (Shin et al., 2016).

## Las Bacterial Quantification and Pathogen Index

Branches showing typical HLB symptoms were tagged for quantification of Las bacterial titers at the beginning of the experiment and were used for sampling throughout the study. Samples of mature leaves (six leaves) from the tagged symptomatic branch were taken at 4-month intervals between April 2015 and December 2017 for a total of nine samples per tree. Samples were kept cool and out of direct sunlight. DNA from these samples was extracted for quantification of the Las bacterial titer. Total 1,728 leaf samples taken from trees in each of the groves were assayed for Las bacterium by qPCR. Cycle threshold (Ct) value was measured by real-time PCR using previously described primer sets and probes (Li et al., 2006). Las bacterial titers were assigned to categories 0 to 4 based on Ct values where: category 0 = Ct ≥36.0; category 1 = 32.0≤Ct<36.0; category 2 = 28.0≤Ct<32.0; category 3 = 24.0≤Ct<28.0 and category 4 = Ct< 24.0. The pathogenic index (PI) used to evaluate Las bacterial titer was calculated for each treatment as follows (Yang et al., 2016b):

$$\mathrm{PI} = \sum_{n=0}^{4} \frac{\text{Sum of all numerical grades}}{\text{Total number of plants counted} \times \text{maximum grade}} \times 100$$

## Tree Scoring and Decline Index

Tree health was evaluated using scores based on the observable amount of HLB decline, also known as decline indexing (DI). The same skilled field manager scored all 192 treated trees at 6 month intervals over 3 years using a 0–4 scale where category 0 = No HLB symptoms, normal fruit load

and size, normal leaf size and growth flushes; category 1 = Some HLB symptoms, modest fruit load and normal fruit size, mostly normal growth flushes; category 2 = Some HLB symptoms, modest fruit load with some smaller fruit sizes, most growth normal; category 3 = Obvious HLB symptoms, light fruit load and multiple small fruit sizes, modest or no growth flushes; category 4 = Obvious HLB symptoms, including small leaves and dead wood, small fruit size and virtually no new growth (see Field Trial Evaluation Methods for Growers at https://citrusrdf.org/wp-content/uploads/2012/10/Citrus-Expo-2016-Slinski.pdf).

The DI was calculated for each treatment as follows:

$$DI = \sum_{n=0}^{4} \frac{\text{Sum of all numerical scores}}{\text{Total number of plants counted} \times \text{maximum score}} \times 100$$

## Psyllid Monthly Counts

Each month the incidence of psyllids in each citrus grove was determined using the tapping method (Qureshi et al., 2014). In each grove, 40 random trees, either per row or per bed, were sampled with four tap samples taken per tree. For each tap sample, one of four randomly chosen branches was tapped three times using a PVC pipe rod and insects were collected on a single sticky card attached to a clipboard placed below the branch or branches. The number of psyllids that landed on the sticky card was recorded for 16 trees and 10 card samples were taken per grove. Significant differences among the groves were determined by ANOVA and *ad hoc* means were compared using a Tukey's test ($\alpha$ = 0.05).

## Statistical Analysis

To identify differences among the rates of increase in Las bacterial titer, Ct values (Ct) for each treatment and Ct data (y) over time were fitted to linear [t = t/y] models, according to Madden's method (Madden and Ellis, 1988). Rates of Ct increase for the superior model were then compared using a *t*-test to determine significant differences. To examine the dynamics of HLB infection, Ct increase (y/t), the rate of Ct increase (dy/dt), and the acceleration/deceleration of the epidemic [(dy/dt)/dt] were calculated for each of the treatments, wherein y=Ct value for each treatment and t=time in months. Linear regression was used to examine the slopes (b) of the [(dy/dt)/dt] data points, wherein the arithmetic sign of the slope, +b and -b, is indicative of the rate of acceleration and deceleration in the epidemic growth rate, respectively. Slope comparisons were made by *t*-tests, wherein $t = (b_1\text{-}b_2)/(SE_{b1}\text{-}SE_{b2})$, and $b_1$ and $b_2$ are the slopes of the regression lines to be compared. $SE_{b1}$ and $SE_{b2}$ are the respective standard errors of the slopes.

To evaluate the effect of each treatment, both PI and DI were used to calculate the efficacy of each treatment (antimicrobials, heat and additional nutrition). Without considering the background effect of the treatment, the absolute therapeutic efficacy ($E_{abs}$) was simultaneously calculated from the DI or PI for each treatment by:

$$E_{abs} = \frac{DI_{ck} - DI_{tr}}{DI_{ck}} \times 100$$

Where $DI_{ck}$ = DI of control; $DI_{tr}$ = DI of treatment

The control increment of the DI ($\Delta_{ck}$) was calculated by:

$$\Delta_{ck} = \frac{DI_0 - DI_T}{DI_0} \times 100$$

Where $DI_0$ = DI of control at pre-treatment; $DI_T$ = DI of control at post-treatment.

To avoid background effects of the treatment, the relative therapeutic efficacy ($E_r$) for each treatment was determined according to the following equation adopted by Rewal and Jhooty (1985):

$$Er = \frac{DI_0 \times (1 + \Delta_{ck}) - DI_t}{DI_0 \times (1 + \Delta_{ck})} \times 100$$

**TABLE 2 |** Intercepts (a) and slopes (b) of Ct (y) over time (t) calculated from linear [*t* = t/y] models for each treatment.

| Chemicals | Grove B | | Grove E | | Grove P | | Slope (b)* |
|---|---|---|---|---|---|---|---|
| | a | b | a | b | a | b | Mean ± *Sd* |
| ALI | 0.0150 | 0.0326 | −0.0198 | 0.0359 | 0.0019 | 0.0324 | 0.0336 ± 0.0020$^{ab}$ |
| CARV | 0.0119 | 0.0347 | −0.0157 | 0.0358 | −0.0022 | 0.0351 | 0.0352 ± 0.0006$^{a}$ |
| CK | 0.0388 | 0.0327 | −0.0023 | 0.0360 | −0.0148 | 0.0372 | 0.0353 ± 0.0023$^{a}$ |
| OXY | 0.0204 | 0.0332 | 0.0064 | 0.0340 | 0.0070 | 0.0307 | 0.0326 ± 0.0017$^{ab}$ |
| PCY | 0.0359 | 0.0324 | −0.0150 | 0.0365 | −0.0068 | 0.0353 | 0.0347 ± 0.0021$^{a}$ |
| PEN | 0.0291 | 0.0294 | 0.0111 | 0.0304 | 0.0170 | 0.0301 | 0.0300 ± 0.0005$^{b}$ |
| SDX | 0.0273 | 0.0323 | −0.0024 | 0.0343 | −0.0147 | 0.0362 | 0.0343 ± 0.0020$^{a}$ |
| SN | 0.0170 | 0.0320 | −0.0067 | 0.0351 | 0.0063 | 0.0327 | 0.0333 ± 0.0016$^{ab}$ |
| SP | 0.0326 | 0.0334 | −0.0131 | 0.0363 | −0.0054 | 0.0340 | 0.0346 ± 0.0015$^{a}$ |
| STREP | 0.0236 | 0.0321 | −0.0273 | 0.0376 | 0.0137 | 0.0312 | 0.0336 ± 0.0035$^{ab}$ |
| VA | 0.0292 | 0.0337 | −0.0101 | 0.0349 | −0.0100 | 0.0333 | 0.0340 ± 0.0008$^{ab}$ |
| ZS | 0.0147 | 0.0349 | −0.0093 | 0.0367 | −0.0143 | 0.0350 | 0.0355 ± 0.010$^{a}$ |

** Different letter showed significance at 0.05 levels.*

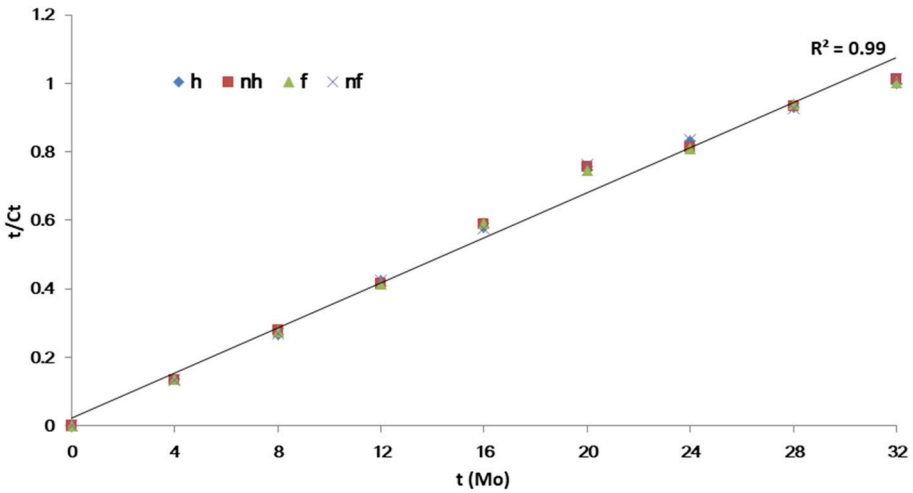


**FIGURE 1 |** Linear [$t$ = t/y] models regression of Ct value (y) over time (t) for heat and additional nutrition treatments.

Where $DI_0$ = DI at pre-treatment; $\Delta_{ck}$ = the control increment of DI; $DI_t$ = DI at post treatment.

# RESULTS

## Titers of Las in HLB-Affected Trees Treated by Integrated Managements

Based on visual observation and qPCR quantification, Grove B had the highest HLB infection rate at 95.4%, followed by Groves E and P with rates of 39.4 and 45.2%, respectively. Variance analysis showed no significant differences in Ct values ranged from 25.1 to 27.7 among the HLB-affected trees at same grove prior to treatments. However, Grove B had significantly lower Ct values (i.e., higher Las titers) compared to Groves E and P. Ct values also varied according to seasonal fluctuations and associated integrated treatments. Ct data (y) over time (32 months) were fitted to linear [t=t/y] models for each treatment. The slopes (b) and intercept (a) in this paper indicated that acceleration/deceleration of the epidemic of HLB. Although the $t$-test showed the slopes for the [(dy/dt)/dt] data points significantly differed among all chemical treatments ($p \leq 0.001$), the intercept of the data points did not ($p > 0.05$). The slope for the positive control PEN treatment was significantly lower than that of the untreated control (CK), followed by OXY, SN, ALI, STREP and VA (**Table 2**). There were no significant differences in the slopes for values derived for trees subjected to heat treatment ($p = 0.885$) and/or given additional nutrition ($p = 0.816$) (**Figure 1**). To lessen the background noise of the treated trees, a pathogen index (PI) was calculated for each chemical compound (**Figure 2**). The PI differed significantly among the groves according to variance analysis (**Figure 2C**). Relative to Groves E and P, Grove B showed a higher PI in the first year after the initial treatment in April 2015, then decreasing to below that of Grove E in the third year. Although no significant difference in PI was found among the chemical treatments in the first year after initial treatment, the differences among chemical treatments later attained significance. PEN had the highest Ct and the lowest PI, and thus was the most effective at eliminating Las, followed by OXY and SN. Compared to the untreated CK, samples from trees treated with ZS, PCY and SDX showed no significant differences in either Ct value or PI.

## Tree Scoring and Decline Index (DI) of the HLB-Affected Trees Treated by Integrated Managements

Grove B had the higher health scores and DI in relative to Grove E and P (**Figures 3C**, **4C**). Higher scores and DI were associated with poorer growth. Differences in the tree scores and DI among the chemical treatments were significant after 1 year post initial application, especially PEN, followed by Oxy and SN. Chemical treatments significantly affected tree health scores 1 year after initial application in all three groves. PEN was the most effective of the chemical compounds, followed by SN and OXY (**Figures 2A–C**, **3A,B**, **4A,B**). Compared to without treatments (NF and NH), heat treatment (H) had a short-term positive effect on tree growth and vigor during the first 18 months, whereas additional nutrition (F) had a cumulatively positive effect on growth and vigor over the 2 years after initial application, especially in Grove B (**Figures 3D,E**, **4D,E**).

# THERAPEUTIC EFFICACY OF THE CHEMICAL COMPOUNDS

The therapeutic efficacies were calculated from PI and DI for each chemical treatment during the period between 2016 and 2018, respectively (**Figure 5**). The average therapeutic efficacy was 0.3% in the first year (2016) after the initial application, then increased up to 20% in 2017 (Year 2) and 2018 (Year 3). In Years 2 and 3, all antimicrobial compounds were effective with therapeutic efficacy values that ranged from 4.2% (SDX) to

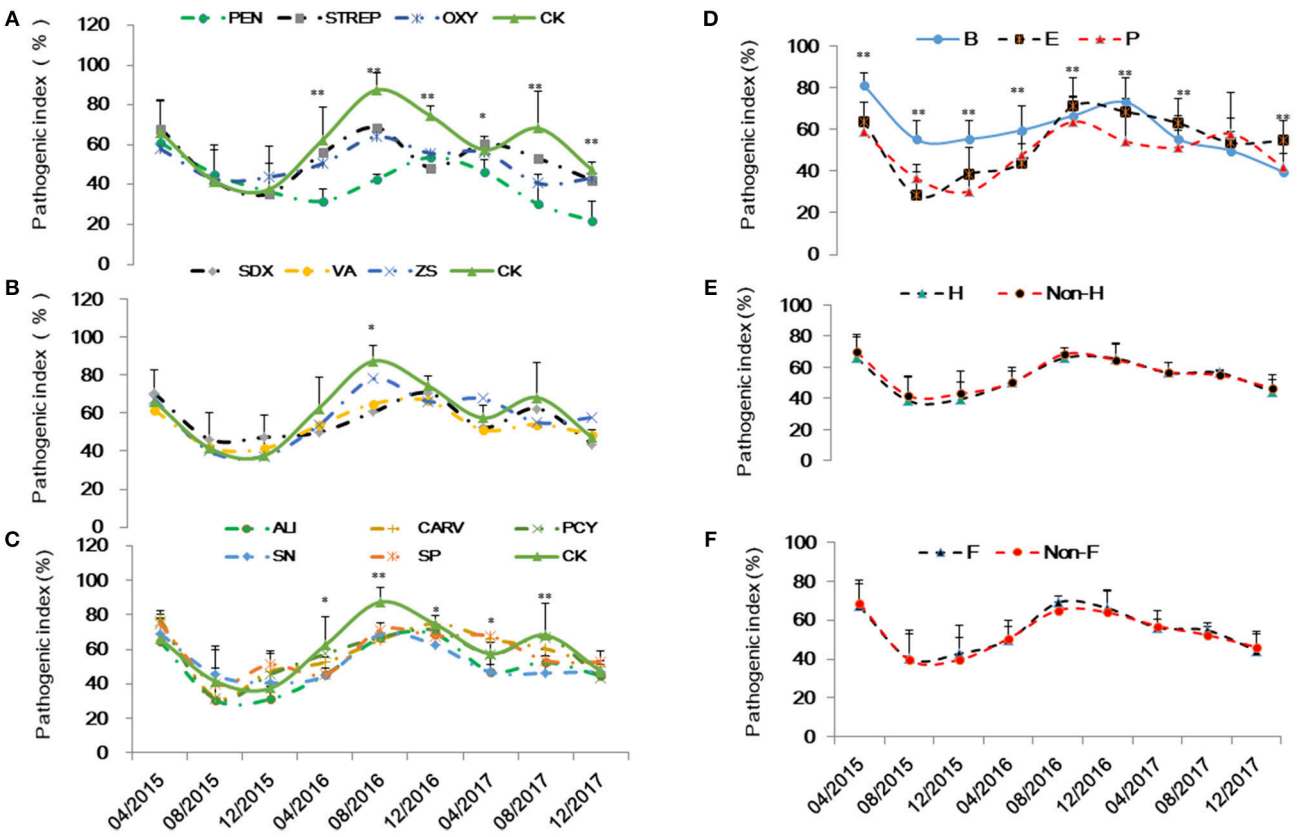


**FIGURE 2 |** Pathogenic index of the integrated management by chemical (**A** and **B**: the abbreviations listed in **Table 1**), grove (**C**: groves **B,E** and P), heat (**D**: H, heat treatment; Non-H, without heat treatment) and additional nutrition (**E**: F, additional nutrition; Non-F, without additional nutrition).

49.3% (PEN) in Grove B. With the exception of SP in Grove E and SDX in Grove P, the other antimicrobial compounds also effectively promoted citrus growth, with therapeutic efficacies ranging from 5.73% (VA) to 65.2% (PEN). PEN increased its therapeutic efficacy by 43.6% (2017) and 60.3% (2018) for all three groves.

## Psyllid Monthly Variations in Three Citrus Groves

A similar number of psyllids per card was obtained for all groves during June 2015 (**Figure 6**), although the actual number of psyllids was higher for Grove B relative to Groves E and P ($F = 94.19$; df = 2, 18; $P < 0.0001$) in July 2015. Between September 2015 and January 2016, psyllids were found only in Grove B. By February 2016, the numbers of psyllids were similar across all three groves, although between March and August 2016 Grove B had more psyllids relative to the other groves. A higher number of psyllids ($F = 4.38$; df = 2, 18; $P = 0.0282$) was seen in Grove B for September 2016, but the psyllid numbers were similar among the three groves between October and December 2016.

In February 2017, after harvest, Grove B had the largest the psyllid population ($F = 5.69$; df = 2, 18; $P = 0.0122$). By March 2017, the citrus tree flowers were blooming, which restricted the spray regime for psyllid control and thus the populations were similar. However, in April 2017 the psyllid population number per tap sample was reduced ($F = 20.4$; df = 2, 18; $P < 0.0001$), with the exception of Grove B, which was similar to the March count. During May 2017, the psyllid population in Groves B and P was similar and higher ($F = 9.55$; df = 2, 18; $P = 0.0015$) than that for grove E. Whereas, the psyllid counts in all groves during June-August 2017 were similar, between September and November 2017, under post-hurricane conditions, the psyllid population significantly differed among the groves. In September 2017 the psyllid mean number was highest ($F = 35.96$; df = 2, 18; $P < 0.0001$) in Grove E. During October 2017, Grove P and Grove E psyllid numbers were similar and lower than that for Grove B ($F = 5.39$; df = 2, 18; $P = 0.0147$). By November 2017, Groves P and B had similar psyllid numbers that were lower than that for Grove E ($F = 3.94$; df = 2, 18; $P = 0.0381$). The mean number of psyllids found per card was similar among the groves during December 2017 and February 2018. No psyllids were found in any grove in January 2018. In nearly all months of the experimental period (June 2015-March 2018) Grove B had a higher mean number of psyllids per card, relative to Grove E and P. Overall, the psyllids were superiorly controlled in Groves E and P compared to that for Grove B.

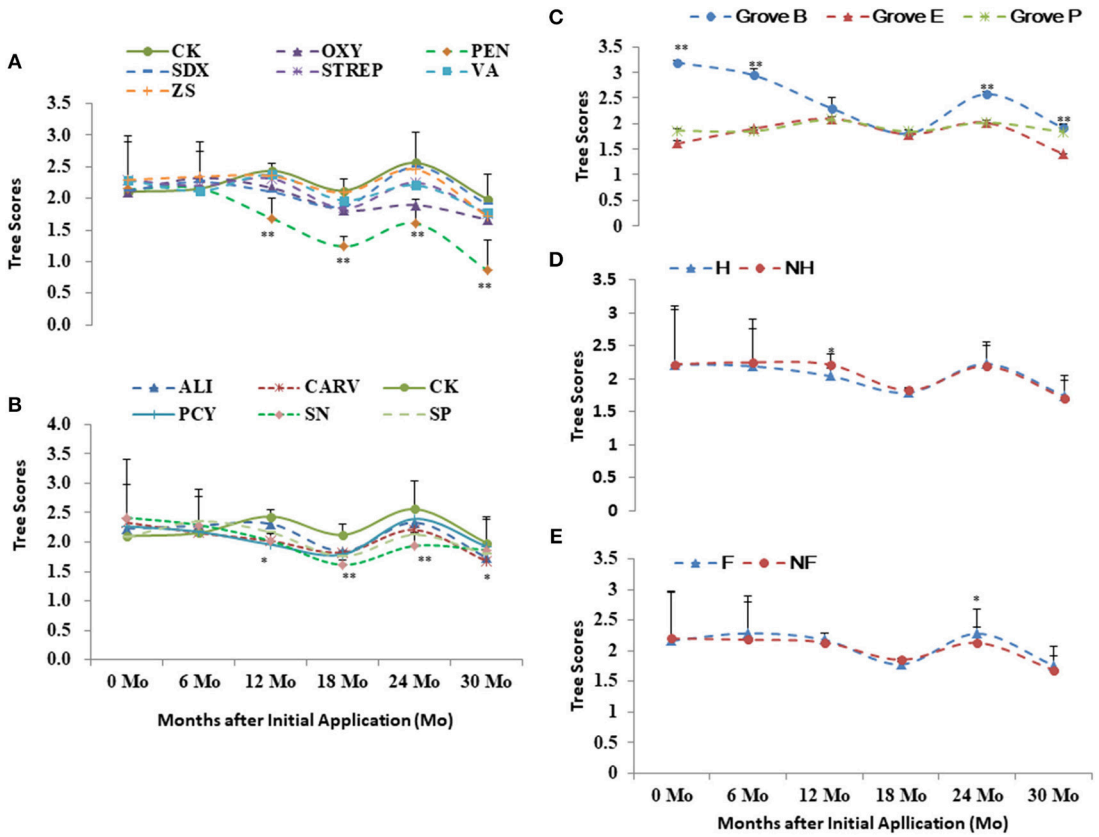


**FIGURE 3 |** Tree scores of the integrated managements treated by chemical (**A,B**: the abbreviations listed in **Table 1**), heat (**D**: H, heat treatment; Non-H, without heat treatment) and additional nutrition (**E**: F, additional nutrition; Non-F, without additional nutrition) in each grove (**C**: groves B, E and P). * and ** significance at 0.05 and 0.01 probability level, respectively.

## DISCUSSION

The citrus industry across the United States relies on finding a sustainable solution that eliminates the spread of HLB and restores HLB-affected trees back to a healthy, productive state through the discovery and application of antimicrobial compounds that either kill or suppress Las. Therefore, our study developed an integrated strategy that includes chemotherapy, thermotherapy and additional nutrition treatment in three different field trials over three consecutive years. To evaluate the complex interaction in the field trial, several analysis methods including the slopes of the dy/dt, the PI and DI from Ct value and tree scores, and the therapeutic efficacies from PI and DI were carried out in this study. Our field plots showed substantial variance in Las bacterial titers (Ct value) that could be due to several factors: (i) an uneven distribution of Las bacterium in the treated trees; and (ii) epidemics occurring in plots subjected to different treatments may peak in terms of HLB incidence or typical symptoms over seasons that may have differing environmental conditions and favorability for citrus HLB (Hu et al., 2017). Taken together, the slope of the Ct value, as well as the PI and DI, reflected the effectiveness of the chemical compounds against Las bacterium in the field. Moreover, the relative therapeutic efficacy is a simple and direct method to evaluate the antimicrobial activity of chemical compounds against Las bacterium that avoids background effects in HLB-affected trees.

In 3-year field trials, several chemical compounds including PEN, OXY calcium complex, silver compounds and Fosetyl-Al, were effective at reducing Las titers in three groves, especially PEN treatment is most effective against Las. Previous reports also showed that PEN is effective in eliminating the Las bacterium and promoting plant growth in graft-inoculated plants (Zhang et al., 2011). PEN is a beta-lactam antibiotic, which binds PEN binding proteins to inhibit cell wall synthesis (Spratt and Cromie, 1988). In addition to its bactericidal effect, PEN can also promote plant growth (Ur Rahman et al., 2004; Zhang et al., 2010).

OXY is short-acting antibiotics that inhibits bacterial growth by inhibiting translation. It passively diffuses through porin channels in the bacterial membrane, binds reversible to the bacterial 30S ribosomal subunit and prevents the aminoacyl tRNA from binding to the A site of the ribosome (Chopra and Roberts, 2001). In the previous studies, OXY can suppress Las titer in greenhouse and field (Schwarz and Van

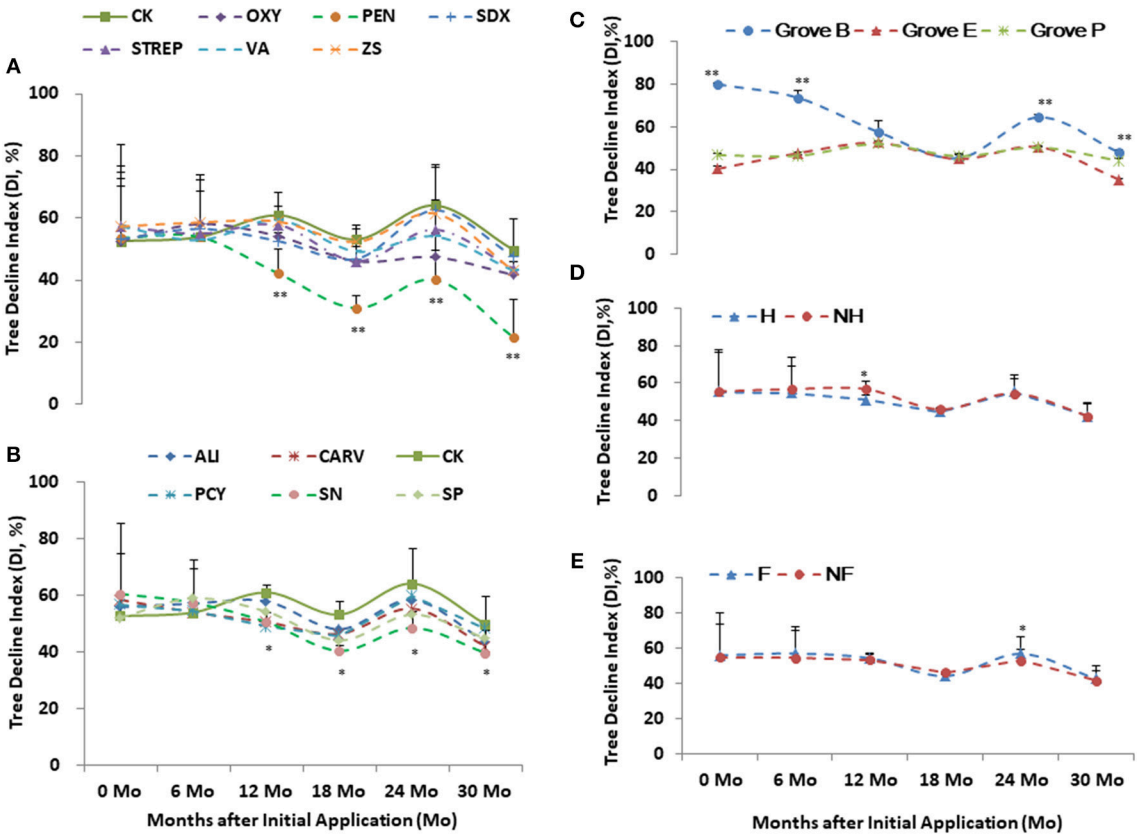


**FIGURE 4 |** Tree decline index (DI) of the integrated managements treated by chemical (**A** and **B**: the abbreviations listed in **Table 1**), heat (**D**: H, heat treatment; Non-H, without heat treatment) and additional nutrition (**E**: F, additional nutrition; Non-F, without additional nutrition) in each grove (**C**: groves B, E and P). * and ** significance at 0.05 and 0.01 probability level, respectively.

Vuuren, 1971; Aubert and Bové, 1980; Hu et al., 2017). In this study, OXY calcium complex was also effective at reducing Las titers by foliar spray in 3-year field trials. Furthermore, under the emergency Exemption provisions of Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Florida has declared an HLB crisis that allows use of OXY calcium complex for controlling citrus HLB by foliar application in Florida.

Silver compounds also exhibit antimicrobial activity toward a range of microbes by altering cell membrane structure and function (Mc Donnell et al., 1999; Pal et al., 2007). As such, silver has been applied to control plant pathogens (Jo et al., 2009; Krishnaraj et al., 2012). These compounds have antimicrobial activity at very low concentrations (<1–10 μmol) that are not toxic to human (Berger et al., 1976), although at higher amounts silver-based compounds can be toxic to mammals as well as freshwater and marine organisms (Bianchini et al., 2002). In this study, silver ions (silver nitrate) showed antimicrobial activity against Las bacterium over 3-year experimental period (**Figure 5**). Therefore, this chemical compound might be a candidate for controlling citrus HLB pathogens under field conditions.

Fosetyl-Al (ALI) is a phosphonate that demonstrated antimicrobial effects when used to treat infections caused by various microorganisms (Darakis et al., 1997; Panicker and Gangadharan, 1999). Several studies demonstrated that the mode of action of phosphonates can alter fungal metabolism (Guest and Grant, 1991) and cell morphology (Jiang, 1990; Jiang and Grossmann, 1992). ALI suppressed Las titers with PI from 64.6% (April, 2015) down to 44.8% (December, 2017) in 3 years of field trials in this study.

Both mobile steam heat treatment and additional nutrition did not promote elimination or suppression of the Las bacterium. Mobile steam heat treatment administered as a 30 s exposure to 51.6–53.3°C had a short-term effect on HLB-affected citrus tree growth in the first year after the initial application, whereas additional nutrition had some cumulative effects on citrus tree growth in the third year after the initial application, especially in the severely HLB-affected citrus Grove B. Notably, one HLB-affected tree that did not have Las bacterial infection in the roots was inadvertently subjected to excessive heat treatment that caused loss of all leaves, but this tree exhibited growth after removal of all affected branches as well as branches having a poor appearance (**Figure 7**). In previous studies, heat treatment

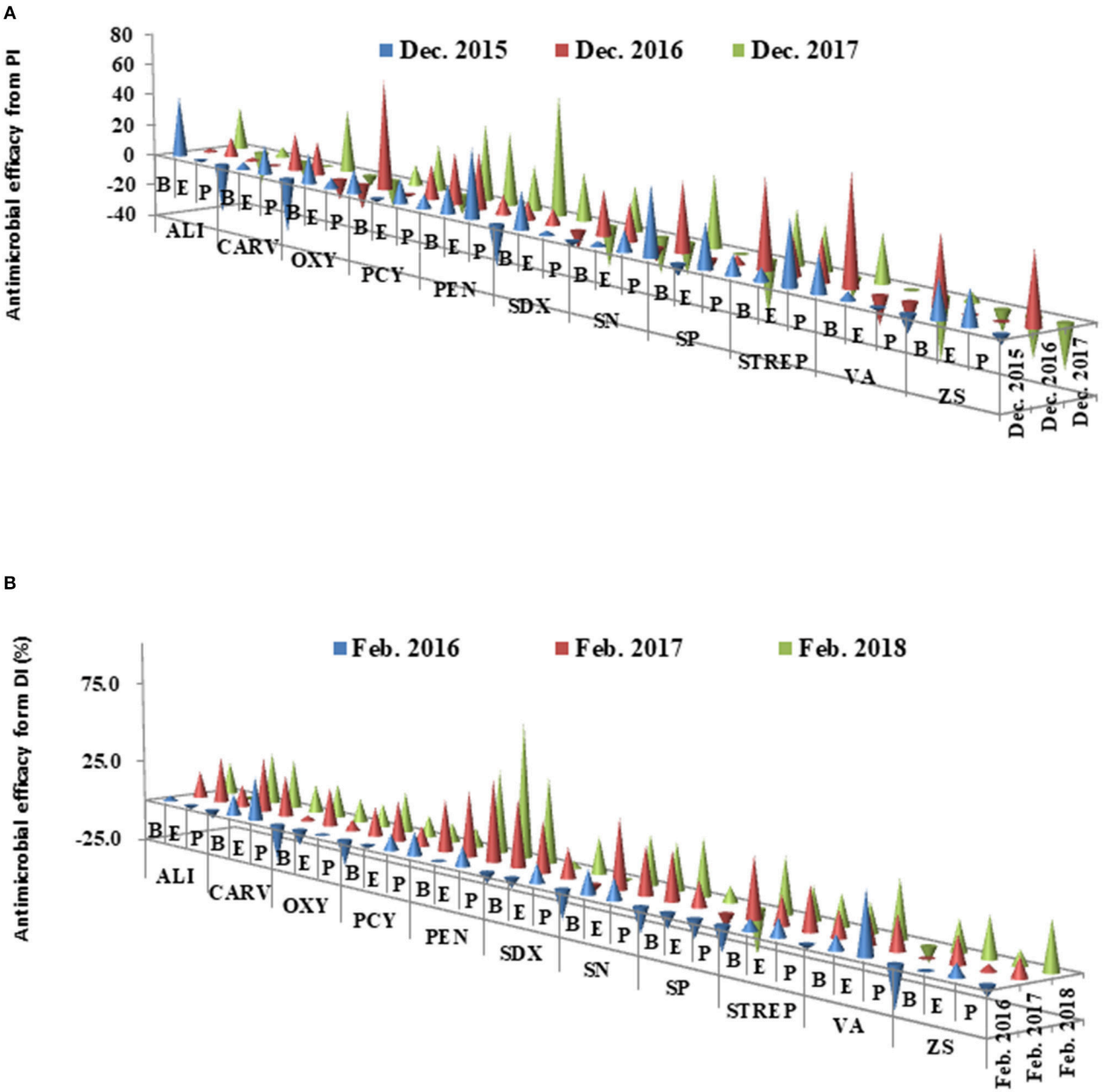


**FIGURE 5 | (A,B)** Antimicrobial efficacies of chemical treatments calculated from pathogenic index (PI) and decline index (DI) of the chemical compounds in three groves from 2015 to 2018. All abbreviations used in this figure were listed in **Table 1**.

enhanced vigor and eliminated Las in HLB-affected citrus (Hoffman et al., 2013; Yang et al., 2016b). Therefore, heat treatment of HLB-affected citrus trees could synergize with chemical compounds.

Foliar micro-nutrient deficiencies are a noted symptom of HLB-affected citrus trees (Spann and Schumann, 2009). Therefore, foliar applications of micro-nutrients have been used by an increasing number of Florida citrus growers to help mitigate HLB-induced deficiencies and counter the debilitating effects of the disease. Our results indicated that nutrition could improve citrus vigor and growth, but was not effective against Las, as previously reported (Stansly et al., 2014). Therefore, nutrition treatment needs to be repeated in control of HLB. However, a combination of heat and nutrition with chemical treatment, especially PEN, SN, and OXY, in an integrated strategy was effective against Las and promoted the growth of HLB-affected citrus trees. This integrated approach may be beneficial for younger planting groves that have low HLB incidence.

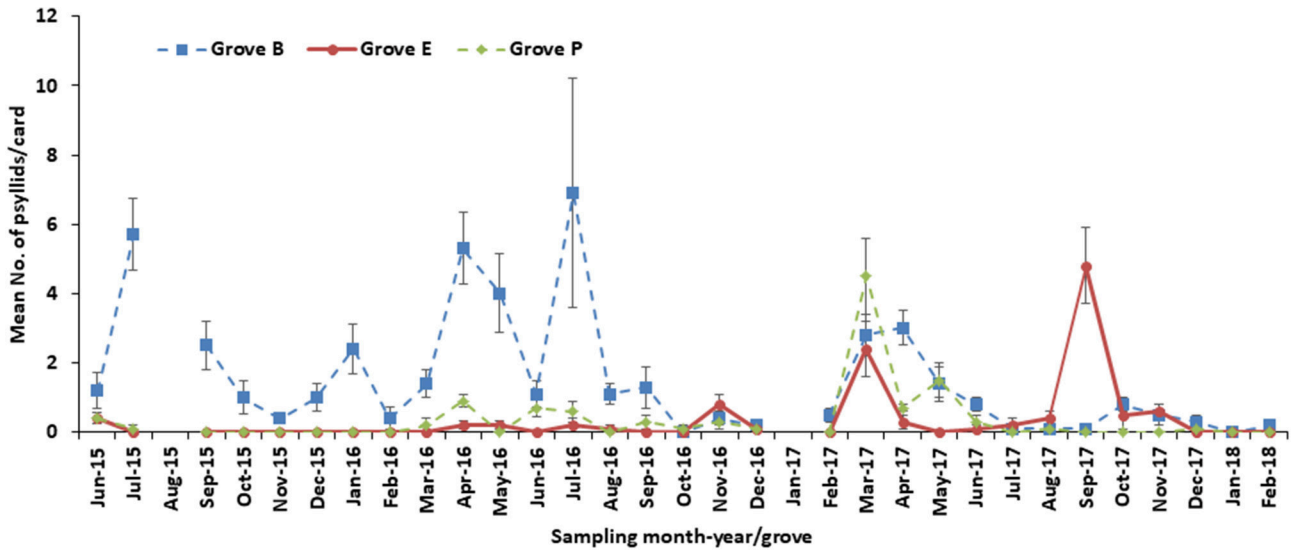


**FIGURE 6 |** Number of psyllid from tap samples in three groves (B, E and P) from 2015 to 2018.

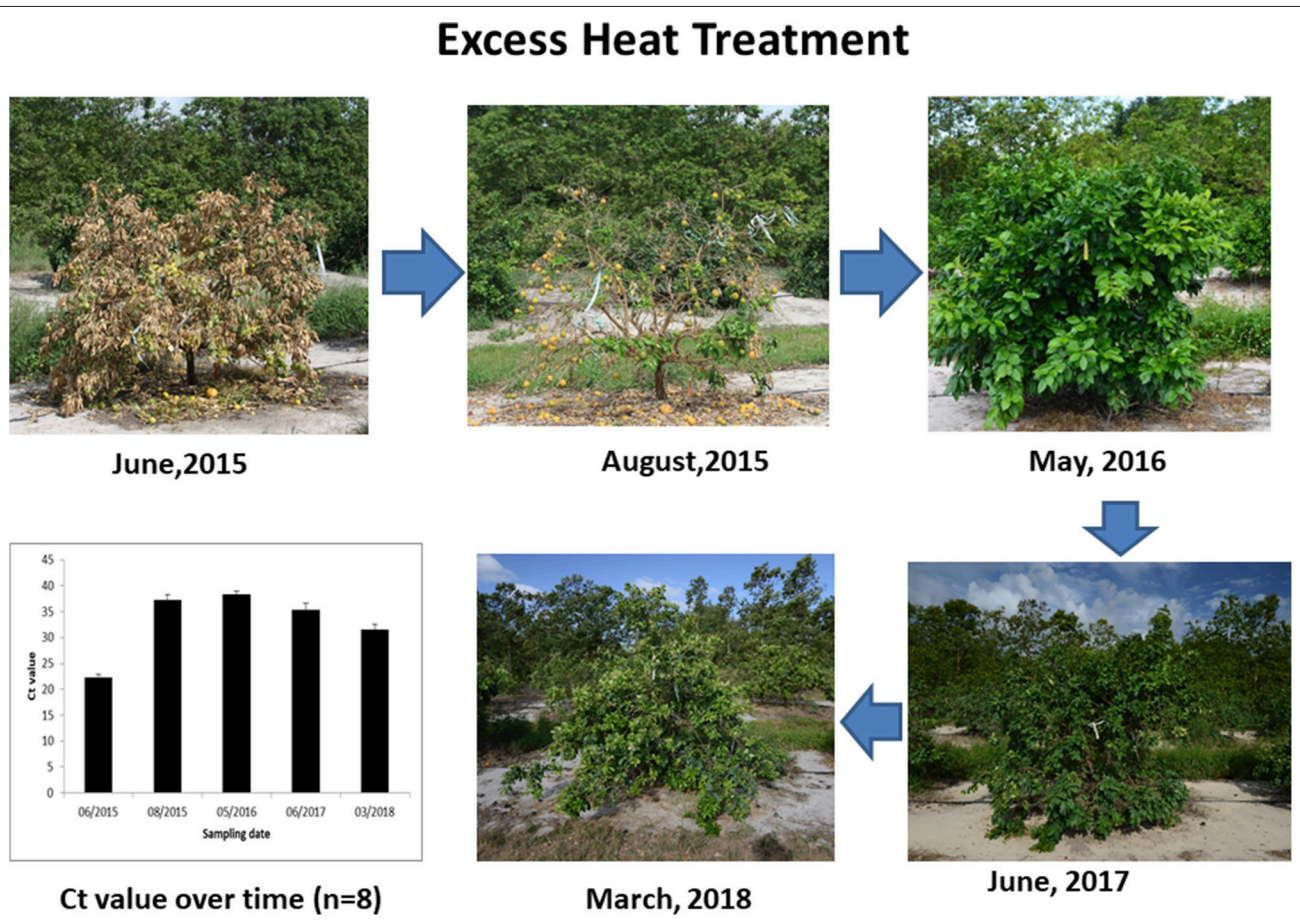


**FIGURE 7 |** Photos and Ct value of HLB-affected citrus trees before and after over heat treatment.

However, novel, environmentally safe chemical compounds for use against Las will still require screening of effectiveness as part of an integrated strategy to combat the incidence of citrus HLB.

## AUTHOR CONTRIBUTIONS

CP, YD, and MZ conceived and designed the experiment. CY, PA, JW, and YH performed the experiments. MZ, PA, and CY analyzed the data. CP and YD contributed reagents, materials, analysis tools. MZ, CY, PA, CP, and YD wrote and revised the paper.

## FUNDING

This work was funded by HLB-MAC Research Grant (8130-0504-CA), NIFA Award No. 2017-70016-26142 and Science and Technology Major Project of Guangxi (Gui Ke AA18118046).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a shared affiliation, though no other collaboration with several of the authors MZ, CY, CP, PA, JW, and YH at time of review.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 20 March 2019
doi: 10.3389/fpls.2019.00277

# Phloem Regeneration Is a Mechanism for Huanglongbing-Tolerance of "Bearss" Lemon and "LB8-9" Sugar Belle® Mandarin

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Huanglongbing (HLB) is an extremely destructive and lethal disease of citrus worldwide, presumably caused by phloem-limited bacteria, *Candidatus* Liberibacter asiaticus (*C*Las). The widespread invasiveness of the HLB pathogen and lack of natural HLB-resistant citrus cultivars have underscored the need for identifying tolerant citrus genotypes to support the current citrus industry's survival and potentially to lead to future natural HLB resistance. In this study, transverse sections of leaf lamina and midribs were examined with light and epifluorescence microscopy to determine anatomical characteristics that underlie HLB-tolerant mechanisms operating among "Bearss" lemon, "LB8-9" Sugar Belle® mandarin, and its sibling trees compared with HLB-sensitive "Valencia" sweet orange. The common anatomical aberrations observed in all *C*Las-infected varieties are as follows: phloem necrosis, hypertrophic phloem parenchyma cells, phloem plugging with abundant callose depositions, phloem collapse with cell wall distortion and thickening, excessive starch accumulation, and sometimes even cambium degeneration. Anatomical distribution of starch accumulation even extended to tracheid elements. Although there were physical, morphological, and pathological similarities in the examined foliage, internal structural preservation in "Bearss" lemon and "LB8-9" Sugar Belle® mandarin was superior compared with HLB-sensitive "Valencia" sweet orange and siblings of "LB8-9" Sugar Belle® mandarin. Intriguingly, there was substantial phloem regeneration in the tolerant types that may compensate for the dysfunctional phloem, in comparison with the sensitive selections. The lower levels of phloem disruption, together with greater phloem regeneration, are two key elements that contribute to HLB tolerance in diverse citrus cultivars.

**Keywords: HLB, citrus greening, anatomy, phloem disruption, replacement phloem, disease tolerance**

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***Edited by:***
*Marcos Antonio Machado, Instituto Agronômico de Campinas (IAC), Brazil*

***Reviewed by:***
*Alberto Martin Gochez, Instituto Nacional de Tecnología Agropecuaria (INTA), Argentina*
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***Specialty section:***
*This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Plant Science*

***Received:*** *31 July 2018*
***Accepted:*** *20 February 2019*
***Published:*** *20 March 2019*

***Citation:***
*Deng H, Achor D, Exteberria E, Yu Q, Du D, Stanton D, Liang G and Gmitter FG Jr (2019) Phloem Regeneration Is a Mechanism for Huanglongbing-Tolerance of "Bearss" Lemon and "LB8-9" Sugar Belle® Mandarin. Front. Plant Sci. 10:277. doi: 10.3389/fpls.2019.00277*

## INTRODUCTION

Citrus Huanglongbing (HLB, previously called citrus greening and yellow dragon disease), an extremely destructive and lethal disease of citrus (Bové, 2006), was discovered in Guangdong province in south China in 1919 (Reinking, 1919). Since first detected in Miami-Dade county of south Florida in 2005 (Halbert, 2005), fruit yields have declined annually, resulting in

substantial economic losses according to the USDA's National Agricultural Statistics Service (NASS) (Ferreira and Perez, 2017). The sharp decline in fruit production is a consequence of the widespread invasiveness of the pathogen, the unavailability of curative treatments, and the lack of HLB-resistant cultivars (Bové, 2006; da Graca et al., 2016; Miles et al., 2017).

The putative pathological agent of HLB, *Candidatus* Liberibacter spp., is a Gram-negative, thin-walled, phloem-limited bacterial genus belonging to α (alpha) subdivision of the class *Proteobacteria* (Jagoueix et al., 1994; Bové, 2006). So far it has not been successfully cultured. Currently, three species are recognized worldwide: *Ca. L. asiaticus* (*C*Las) (Bové, 2006), *Ca. L. africanus* (CLaf) (Jagoueix et al., 1994), and *Ca. L. americanus* (CLam) (Teixeira et al., 2005), on the basis of their eco-geographic range, transmission vector, and adaptation to warmer or cooler environments. In Florida, *C*Las is the only pathogen identified (Wang and Trivedi, 2013; Brodersen et al., 2014), with primary transmission by the Asian citrus psyllid (ACP, *Diaphorina citri*) (Bové, 2006), though secondary transmission is possible by grafting and dodder (*Cuscuta pentagona*) (Zhou et al., 2007; da Graça, 2008). Foliar asymmetrical chlorosis and blotchy mottle appearance is the most recognized characteristic of HLB symptomatology, which also includes yellow foliage and shoots, leaf loss and fruit drop, stunting and twig dieback, premature and lopsided fruits, and eventual tree death in some, but not all, situations (Aritua et al., 2013; da Graca et al., 2016). Recently, it has been reported that HLB has swept through almost 100% of commercial citrus groves in Florida (Browning, 2015), and more than 80% of all citrus trees have been affected (Albrigo and Stover, 2015).

Previous evidence indicates that *C*Las always resides and colonizes the sieve tubes within citrus phloem tissue (Jagoueix et al., 1994; Kim et al., 2009; Aritua et al., 2013; Wang et al., 2017), which is responsible for carrying photosynthates from source-to-sink in plants (Heo et al., 2014). It is this very tissue that contains the essential and massive nutrient-rich components that support the life activities of both *C*Las and ACP (Hijaz et al., 2016). At the anatomical level, citrus leaf tissue exhibited conspicuous changes induced by *C*Las infection compared to healthy foliage. Ultrastructural examination of tissue from *C*Las-inoculated sweet orange [*Citrus sinensis* (L.) Osbeck] and grapefruit (*C. paradisi* MacFadyen) revealed the early histological symptomatology of middle lamella swelling between cell walls around sieve elements (Folimonova and Achor, 2010), also described as phloem necrosis (Schneider, 1967; Achor et al., 2010). Phloem necrosis is usually accompanied by phloem sieve element plugging from abundant callose and phloem protein 2 deposition (Achor et al., 2010; Koh et al., 2012; Albrigo et al., 2014), followed by phloem cell wall distortion and sieve element collapse (Etxeberria and Narciso, 2015). Because of these phloem disruptions, transport of photosynthates is severely obstructed (Aritua et al., 2013; Albrigo et al., 2014; Etxeberria and Narciso, 2015), which in turn may be responsible for the accumulation of abnormally large quantities of starch granules in virtually all living cells of the aerial organs (Schneider, 1968; Etxeberria et al., 2009), including phloem parenchyma cells and sieve elements (Folimonova and Achor, 2010; Gonzalez et al., 2012).

Currently, there are no commercial citrus cultivars, varieties or scion-rootstock grafting combinations with natural resistance to *C*Las infection (da Graca et al., 2016; Wang et al., 2017). However, a few commercial varieties such as lemon [*C. limon* (L.) Burm. F.] and Persian lime (*C. latifolia*), along with US-897 rootstock (*C. reticulata* Blanco × *Poncirus trifoliata* L. Raf.) and the "LB8-9" Sugar Belle® mandarin hybrid (SB; "Clementine" mandarin × "Minneola" tangelo) (Gmitter et al., 2010) have shown apparent HLB tolerance under Florida natural HLB-endemic conditions (Albrecht and Bowman, 2011; Ramadugu et al., 2016; Stover et al., 2016; Killiny et al., 2017; Miles et al., 2017; Wang et al., 2017). SB mandarin and "Bearss" lemon trees in various locations in Florida maintain vigorous growth, and fruit yield is not significantly affected by *C*Las infection (Gmitter et al., unpublished data). "Valencia" sweet orange (*C. sinensis* (L.) Osbeck) is a well-known HLB-sensitive cultivar (Folimonova et al., 2009). LB8-1, LB8-2, LB8-15, and LB9-13 mandarins are the siblings of SB mandarin; however, they have been found to be very sensitive to HLB, based on more than 10 years of observation (Gmitter et al., unpublished data). Orange, mandarin, and lemon represent three different kinds of citrus from the taxonomic point of view (Wu et al., 2018). Taken together, these similarities and differences in the botanical origin and the range of sensitivity to HLB provide an excellent opportunity to identify factors impacting HLB tolerance.

With overall citrus production sharply down in the United States and other major citrus-producing countries worldwide (Ferreira and Perez, 2017), identifying the presence of HLB tolerance in citrus germplasm resources is highly needed, crucial to the current citrus industry's survival, and is indispensable for future ultimate natural HLB resistance (Killiny et al., 2017; Miles et al., 2017). In the present work, we conducted a comparative pathological and anatomical investigation by sectioning the lamina and midribs and examination by light and epifluorescence microscopy to understand what interior structure makes "Bearss" lemon and SB mandarin tolerant and others sensitive to *C*Las infection in the natural field environment. Results from this study may provide a very useful supplement to current HLB tolerant citrus germplasm knowledge. We hope that in future work, these observations and HLB tolerance mechanism can be used to modify and create citrus germplasm that is more tolerant or ultimately resistant to HLB.

## MATERIALS AND METHODS

### Plant Materials

For basic anatomical studies, fully expanded and hardened leaves of spring flushes with typically visual blotchy mottle symptoms were sampled at the end of July from "Bearss"

lemon, SB mandarin, "Valencia" sweet orange, and siblings of SB mandarin (LB8-1, LB8-2, LB8-15, and LB9-13). SB mandarin and siblings and "Valencia" sweet orange trees were grown in an experimental field of UF-CREC, and "Bearss" lemon trees were grown in a commercial orchard near Vero Beach, Florida. All trees were naturally exposed to HLB disease for at least 10 years. The experimental trees were approximately 20 years old. Correspondingly, HLB-free control SB mandarin samples were collected from a secured screen-house at CREC, which was thoroughly protected from infection and exposed to the same natural environment with those HLB-affected trees. The foliar samples were randomly selected from mature spring flushes with similar physical age and HLB status (**Figure 1**). Before sampling, the trees were assessed by real-time qPCR (quantitative polymerase chain reaction) to confirm CLas infection, according to Li et al. (2006).

## Sample Preparation

The leaves were collected between 9:00 and 10:00 am on a sunny day. Leaf midrib tissue was dissected into 2–3 mm segments with single edged disposable blades and placed immediately into fixative solution of 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M Sorensen's phosphate buffer (pH 7.2) to preserve structure. The tissue was fixed at a minimum of 4 h at RT (room temperature), or overnight at 4°C to adequately infiltrate the specimens. Once fixed, the samples were thoroughly rinsed using the above buffer. Paraffin wax is water immiscible. Therefore, the specimens were dehydrated with ethanol solutions of an

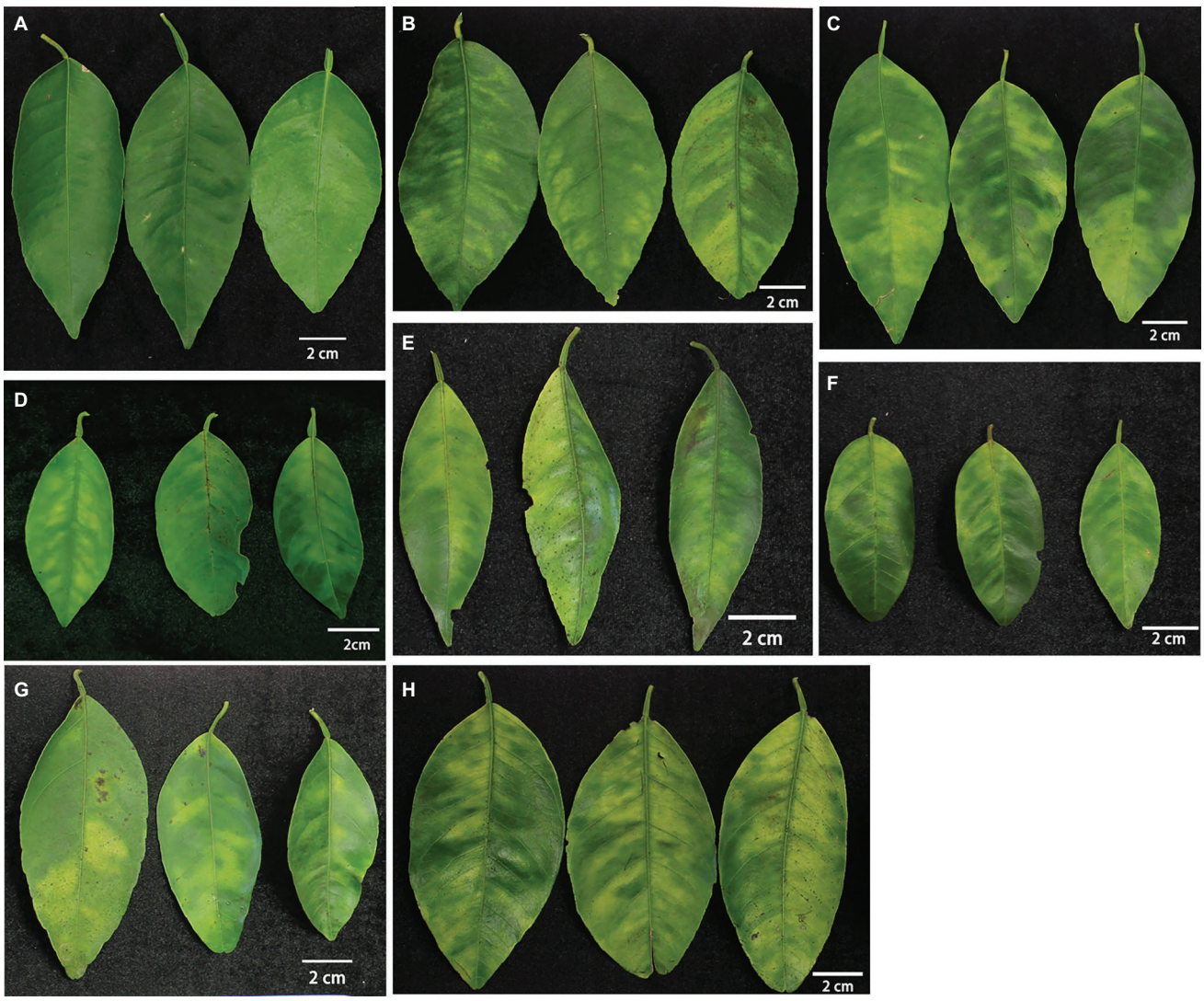


**FIGURE 1 |** The materials used in this study. **(A)** HLB negative and greenhouse-grown SB mandarin; **(B–H)** blotchy mottle leaves from **(B)** "Bearss" lemon; **(C)** SB mandarin; **(D)** "Valencia" sweet orange; **(E)** LB8-1 mandarin; **(F)** LB8-2 mandarin; **(G)** LB8-15 mandarin; and **(H)** LB9-13 mandarin. Notes: LB8-1, LB8-2, LB8-15, and LB9-13 mandarin are siblings of SB, having common parents, "Clementine" mandarin (*C. reticulata* Hort. ex Tanaka) × "Minneola" tangelo ["Duncan" grapefruit (*C. paradisi* Mac.)] × ["Dancy" tangerine (*C. reticulata*)]. The leaves of each cultivar were captured by digital camera with a scale bar of 2 cm.

increasing concentration (30, 50, 70, 85, 95–100% ethanol) to remove both bound and free water. Ethanol changes were made every hour. The successive incremental concentration was used to avoid exorbitant distortion of the specimens.

To further dehydrate the tissue, three changes of increasing ratio of TBA (tertiary buty1 alcohol, also called tert-butanol) were used to displace the ethanol in the tissue (3:1, 1:1, 1:3—ethanol: TBA). Tissue was rinsed in 100% TBA for an hour. TBA was gradually replaced with previously melted paraffin wax using a similar ratio (3:1, 1:1, 1:3—TBA: Paraffin) in an oven at 58°C. Solution changes occurred every 8–14 h. Once transitioned to 100% paraffin, an additional three paraffin changes were made every 24 h for optimal infiltration. Tissue was embedded in 100% paraffin using embedding base mold in the desired orientation. Paraffin was allowed to harden at room temperature and then molds were incubated at 4°C overnight. Paraffin blocks were stored at room temperature. The blocks were then sectioned using a Leica 2155 microtome. 10 μm sections were cut to form a ribbon. Sections were placed on a drop of water on a microscope slide and placed on a hot plate at 37°C hotplate overnight to ensure firm adhesion of sections to the slides.

## Methylene Blue-Azure II-Basic Fuchsin Staining

Slides were dewaxed using three changes of Histoclear II for 15 min each, followed by rehydration through a series of decreasing ethanol washes (100, 70, 30%, 5 min per wash). Slides were rinsed in ultrapure water before staining by methylene blue-azure II-basic fuchsin (Humphrey and Pittman, 1974). The procedures were optimized to stain with methylene blue/azure A for 45 s and counterstaining with basic fuchsin for 30 s, followed by a thorough rinse with distilled water. Slides were dehydrated in an ethanol series (30, 70, 100%) for 3 min each and then cleared in three changes of Histoclear II. A drop of Cytoseal mount was placed over the sections, and a coverslip was placed on the slide. Images were captured using an OMAX 14.0MP CCD digital camera attached to an Olympus BX61 epifluorescence microscope (Olympus Inc., Tokyo, Japan).

## Data Analysis

The scale bars were also captured under the same magnification with samples as reference for the following phloem strip width standardization. The phloem and replacement phloem strip width were quantitatively measured using Image J software (National Institutes of Health, Wayne Rasband, Maryland, USA). The ratio of replacement phloem is calculated by dividing the width of replacement phloem by the total phloem width of the maximal portion within the same leaf. The same position of all the leaves was measured. The result of each leaf was the average of three times technical replicates. The ratio of replacement phloem of each cultivar was based on the average of at least 35 leaves. Minitab version 17 software was used for the means and standard errors calculation. Differences within cultivars were statistically evaluated by one-way ANOVA analysis (analysis of variance). Subsequent comparisons were made using Tukey's HSD test with 95% confidence interval.

# RESULTS

## Comparative Anatomical Changes of HLB-Affected Citrus Lamina

The transverse sections of the lamina internal structures are presented in **Figure 2**. Based on many repeated microscopic observations, there are basic anatomical similarities in the lamina common in HLB-affected and HLB-free citrus leaves (**Figure 2**). The internal structure is protected by an upper and lower epidermis, which typically consists of one thick and compressed uniseriate cell layer. Anatomically, the epidermis is covered by a thick layer of slightly undulate cuticle on the adaxial and abaxial surface. The stomata are found on the abaxial surface. Directly below the epidermis, there stand in parallel columns two or three elongated and pillar-shaped layers of photosynthetic palisade parenchyma cells. Beneath the palisade parenchyma cells lies the loosely packed and irregularly shaped spongy parenchyma cells. Some visible veins, composed of vascular tissue with the xylem above the phloem, are found immersed in lamina parenchyma cells (**Figure 2A**).

HLB-free control SB mandarin possessed very little or nonexistent starch accumulation in lamina internal structure (**Figure 2A**). Among the HLB-affected citrus varieties, however, the lamina of all cultivars displayed starch accumulation but with differing severity. The starch grains were scattered rather sporadically and rarely in the epidermal cells of "Bearss" lemon and SB mandarin lamina (**Figures 2B,C**); however, they were observed as a continuous layer settled to the bottom of the adaxial epidermis in HLB-sensitive "Valencia" sweet orange and SB mandarin siblings (**Figures 2D–H**, note the conspicuous starch grain layer in LB8-2, LB8-15, and LB9-13 mandarins in **Figures 2F–H**). A number of large, spherical or oblate spheroid starch granules entirely filled the whole volume of most palisade parenchyma cells, and the spongy parenchyma cell matrix in "Valencia" sweet orange (**Figure 2D**) and SB mandarin siblings (**Figures 2E–I**). Few and partially-filled starch granules, by contrast, were observed in "Bearss" lemon and SB mandarin mesophyll cells (**Figures 2B,C**).

## Pathological Anatomy Modifications of Citrus Midribs Induced by HLB

Microscopic examination of the transverse sections of the prominent midribs of citrus leaves is presented in **Figure 3**. From the adaxial to abaxial side of the midrib, collenchyma tissue occasionally was found below the upper epidermis. The largest vascular bundle is embedded in the central space with xylem being positioned toward the inner boundary and phloem toward outer boundary, which run parallel to each other and are surrounded by an incomplete sclerenchyma ring (phloem fibers) with heavily thickened cell walls. Xylem

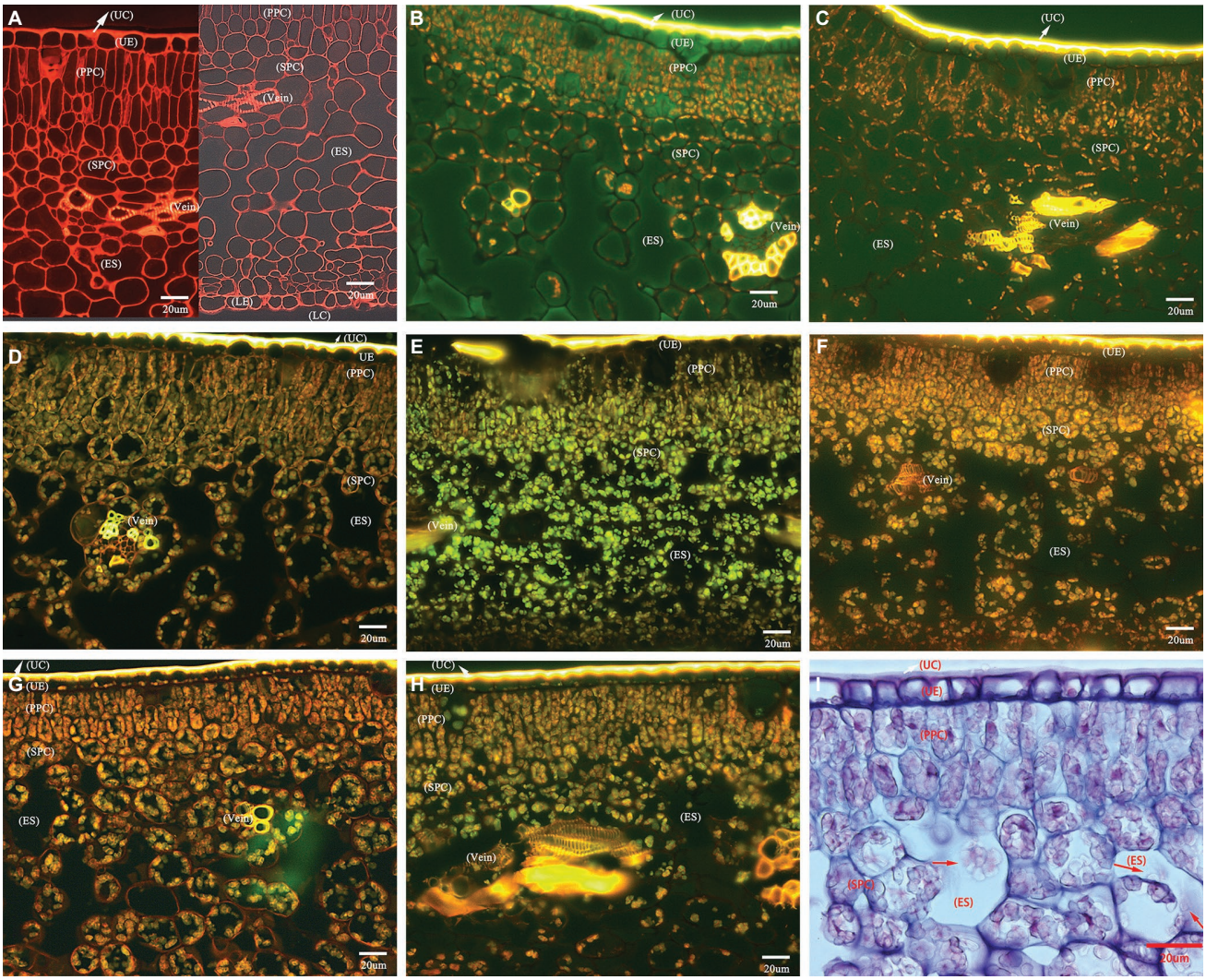


**FIGURE 2 |** Photomicrographs of transverse section showing comparative anatomical changes of HLB-affected citrus lamina. **(A–H)** Epifluorescence micrograph of anatomical characters through 20× objective lens. **(A)** HLB-free SB mandarin; **(B)** "Bearss" lemon; **(C)** SB mandarin; **(D)** "Valencia" sweet orange; **(E)** LB8-1 mandarin; **(F)** LB8-2 mandarin; **(G)** LB8-15 mandarin; **(H)** LB9-13 mandarin; and **(I)** light micrograph through 40× objective lens with methylene blue-azure A solution and basic fuchsin solution staining, LB8-15 mandarin. The red arrow in image **(I)** indicates the starch granules in a light micrograph. Scale bars = 20 μm. Abbreviations: UC, upper cuticle; UE, upper epidermis; PPC, palisade parenchyma cell; SPC, spongy parenchyma cell; ES, intercellular air empty space; LE, lower epidermis; and LC, lower cuticle.

tissue is composed of vessel elements (large), tracheid elements (small), and parenchyma cells (**Figure 3A**). Within phloem tissue, the parenchyma cells can be easily recognized with the largest cell size. Each phloem sieve element (SE) is associated with a very small and neighboring companion cell (CC) that supports the functioning of SEs (**Figure 3B**, white and yellow arrows, respectively).

Compared to the basic structure of foliar phloem tissue from healthy trees, the anatomical aberrations observed in the foliar phloem cells of HLB-affected SB trees are as follows: a few phloem cells plugged with abundant callose depositions (**Figure 3D**, yellow arrows), phloem collapse with cell wall distortion and thickening (**Figure 3D**, blue arrows), and starch accumulation (**Figures 3C,D**, red arrows). Before metaphloem elements are entirely collapsed, SB mandarin showed obvious phloem regeneration (**Figure 3D**, the phloem regeneration was indicated by green line).

## Differential Anatomical Changes in Foliar Phloem of HLB-Affected Citrus Cultivars

Although all the examined midribs came from spring flush leaves of similar age and *C*Las infection, confirmed by qPCR, the severity of anatomical aberrations induced by *C*Las infection varied. On the basis of comparison to the normal parenchyma cells that have smooth edges, "Valencia" sweet orange and SB mandarin siblings suffered more severe cell necrosis, which was characterized

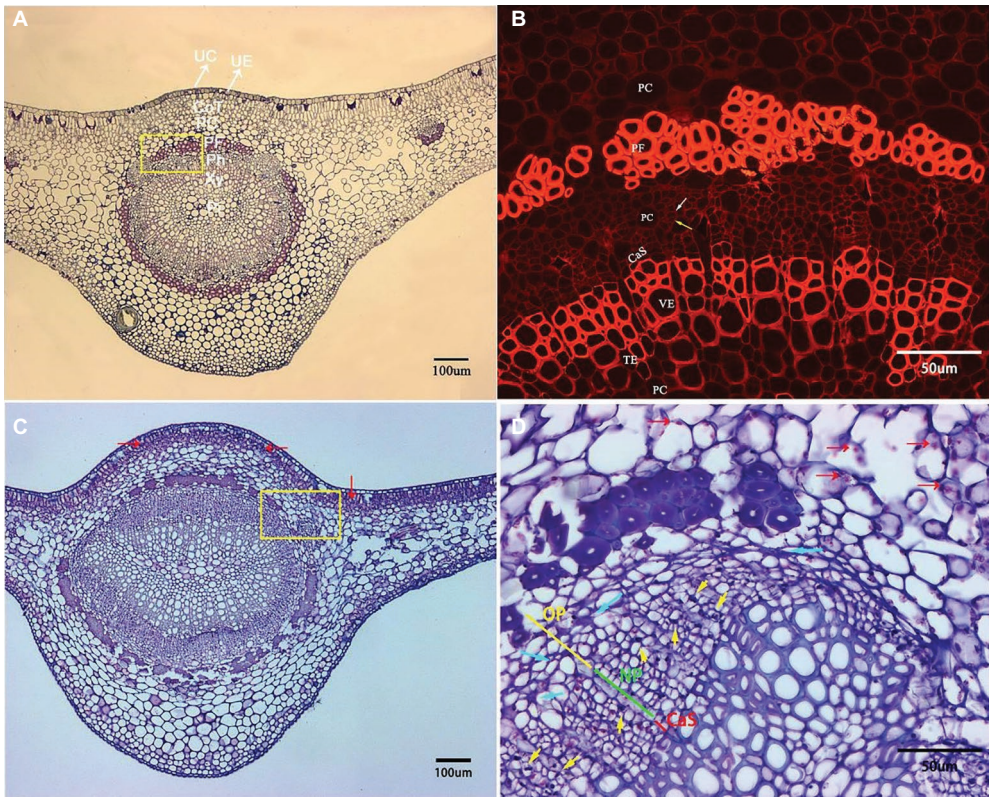


**FIGURE 3 |** Transverse section micrograph of leaf midrib tissue from healthy and HLB-affected SB mandarin tree. Midrib section was observed, and photographs taken under epifluorescence **(B)** and light microscopy **(A,C,D)**. **(B)** and **(D)** are the magnification of the yellow rectangular frame in **(A)** and **(C)**, respectively. **(A,B)** HLB-free SB mandarin. The white arrow indicates the sieve tube elements, and the yellow arrow indicates the companion cell. Healthy phloem cells have smooth edges, thinner cell walls, and lack starch grains. **(C,D)** HLB-affected SB mandarin showing phloem plugging with abundant callose depositions (yellow arrows), phloem collapse with cell wall distortion and thickening (blue arrows), and starch accumulation (red arrows). The green line indicates the replacement phloem, the yellow line indicates peripheral old phloem, and the red line indicates cambium strip. Abbreviations: UC, upper cuticle; UE, upper epidermis; CoT, collenchyma tissue; PC, parenchyma cell; PF, phloem fibre; Ph, phloem; Xy, xylem; Pi, pith; CaS, cambium strip; VE, vessel elements; TE, tracheid elements; NP, new phloem also called replacement phloem in this manuscirpt; and OP, peripheral old phloem. Scale bars = 100 μm **(A,C)**; scale bars =50 μm **(B,D)**.

by excessively swollen or hypertrophic phloem parenchyma cells. The hypertrophic parenchyma cells of "Valencia" sweet orange and SB mandarin siblings became abnormally enlarged or misshapen to giant cells (see the red arrows in **Figures 4C–G**), while the phloem parenchyma cells of "Bearss" lemon and SB mandarin remained largely unchanged (**Figures 4A,B**).

Phloem plugging is one of the anatomical modifications and disorders in sieve elements of all the examined HLB-affected cultivars (**Figures 3D, 4, 5**). Amorphous plugging was not observed in HLB-free control tissues (**Figure 3A**). The dark blue or black densely staining spots lying toward the inner side of sieve tubes seen in the light micrographs, resulting from methylene blue-azure II-basic fuchsin staining, represent the phloem plugging with callose deposition (**Figures 3D, 4**, yellow arrows; **Figures 5A,C**, blue arrows). There were significant amounts of callose deposition located in the sieve elements of new or replacement phloem, or adjacent to cambium regions, in all the cultivars (**Figures 4, 5A**,**C**). Callose depositions were identically confirmed by the epifluorescence micrograph observations by the appearance of red fluorescence spreading prolifically in sieve elements of replacement phloem or adjacent to cambium regions (**Figures 5B,D**). Some of the sieve elements were entirely covered with an abundance of amorphous callose deposition (**Figures 5A,B**).

Phloem collapse with cell wall distortion and thickening is much more frequently observed in "Valencia" sweet orange (**Figures 4C, 5A**) and SB siblings (**Figures 4D–G**). Peripheral old sieve tube members and companion cells collapsed into darkly stained strips and chromophilic masses resembling a solid barrier oriented tangentially, radially, or irregularly between adjacent phloem cells (**Figure 5**). Even at higher magnifications of 20× objective lens, the individual cells are not easily distinguished because of the appearance of dark purple or blue strips of partial or total phloem collapse and disintegration (**Figures 4C–G**). Among the collapsed phloem cells, sieve elements and companion cells were significantly collapsed, while phloem parenchyma cells were expanded to a larger size (**Figure 5**). The amount of collapsed phloem in "Valencia" sweet orange and SB siblings was much greater than that in "Bearss" lemon or SB mandarin (**Figures 4, 5**). By contrast, no phloem collapse was seen in the midribs of HLB-free SB mandarin (**Figure 3A**).

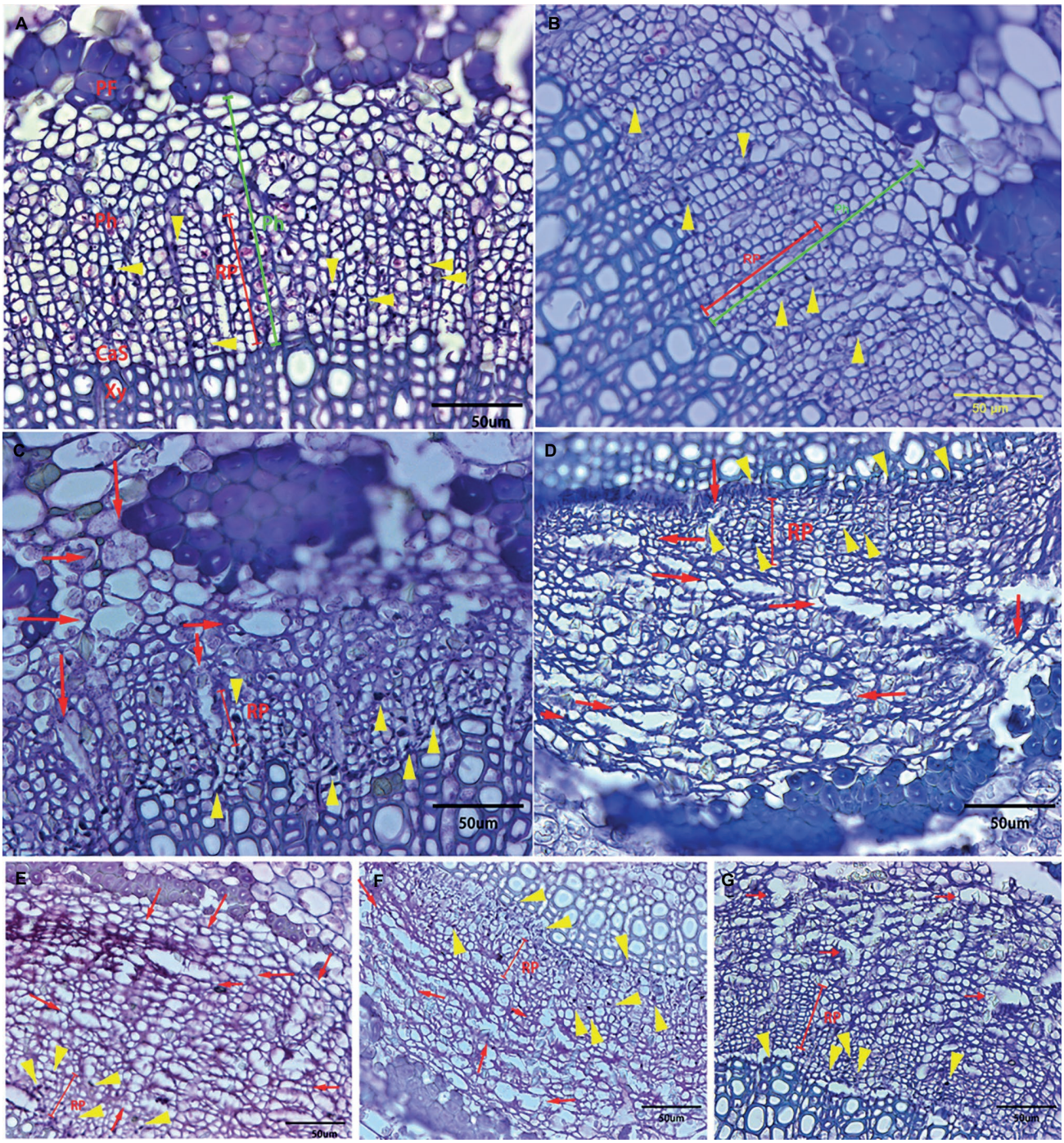


**FIGURE 4 |** Transverse section showing anatomical changes of leaf midrib in response to CLas infection among different citrus cultivars. Midrib section was observed and photographs taken under light microscope. **(A)** "Bearss" lemon; **(B)** SB mandarin; **(C)** "Valencia" sweet orange; **(D)** LB8-1 mandarin; **(E)** LB8-2 mandarin; **(F)** LB8-15 mandarin; and **(G)** LB9-13 mandarin. The red arrow indicates the hypertrophic parenchyma cells that became abnormally enlarged or misshapen to giant cells. The callose-plugged sieve elements appeared as densely stained blue or black spots as indicated by yellow arrows. The red line segment indicates the replacement phloem. The green segment indicates the phloem. Abbreviations: PF, phloem fibre; Ph, phloem; CaS, cambium strip; RP, replacement phloem; and Xy, xylem. Scale bar = 50 μm.

A particularly intriguing result is that, as the peripheral old phloem died or began collapsing in CLas-infected leaves of all the cultivars, some young replacement phloem was generated directly adjacent to the undifferentiated vascular cambium zone as shown above in **Figure 3D**. Compared to the normal phloem, the newly generated replacement phloem was generally composed of sieve elements, companion cells, and phloem parenchyma cells, but neither phloem fibers nor sclereids were observed.

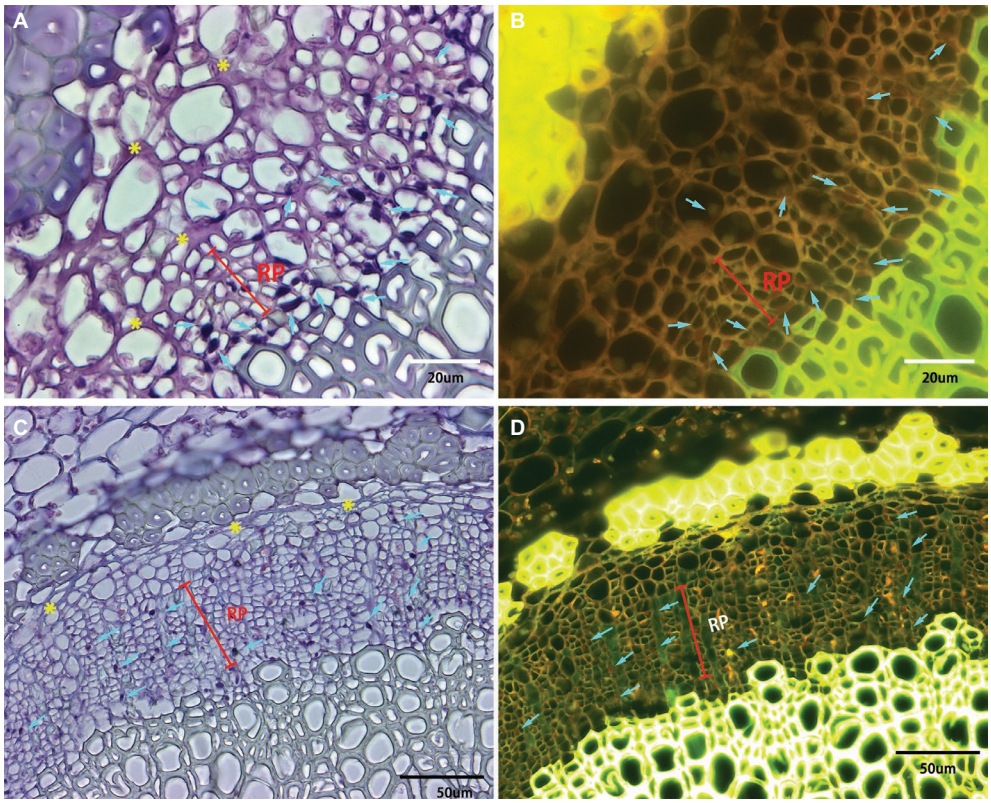


**FIGURE 5 |** Light and epifluorescence photomicrographs of phloem plugging and collapse. Phloem plugging by callose deposition was marked by the light blue arrows with the dark blue or black densely staining spots lying toward the inner side of sieve tubes. The darkly stained strip resembling a solid barrier oriented tangentially, radially, or irregularly between adjacent phloem cells represented phloem collapse (indicated by yellow asterisks). **(A,B)** Valencia sweet orange, through 40× objective lens, **(C,D)** Sugar Belle mandarin, through 20× objective lens. Note a high accumulation of starch in the parenchyma cells and phloem cells. Scale bars = 20 and 50 μm.

The size of replacement phloem was smaller than the peripheral normal phloem (**Figures 3D, 4, 5**).

Statistically speaking, SB mandarin (**Figure 4B**) and "Bearss" lemon (**Figure 4A**) had the largest replacement phloem ratio of 67.37 and 65.73%, respectively, which was significantly higher ($p < 0.05$) than in HLB-sensitive cultivars (**Figure 6**). Viewed in transverse section among all the examined samples, "Bearss" lemon (**Figure 4A**) and SB mandarin (**Figure 4B**) had the largest replacement phloem band and cell layer indicating greater activity of the vascular cambium. The replacement phloem in "Bearss" lemon (**Figure 4A**) and SB mandarin (**Figure 4B**) likely enables a prolonged actively functioning phloem tissue, with very little necrosis and collapse. Although SB siblings and "Valencia" also had the replacement phloem layer (**Figures 4, 6**), their newly generated replacement phloem cells become necrotic and showed typical anatomical alterations of HLB disease as described above (**Figures 4C–G**). Although SB siblings had the largest width of total phloem layer, their newly generated replacement phloem cells become necrotic and showed typical anatomical alterations of HLB disease as described above (**Figures 4D–G**). Valencia sweet orange had a narrowest ring of newly generated replacement phloem (15.19%) (**Figure 6**), and the degeneration of replacement phloem is most pronounced (**Figure 4C**).

Similar to lamina, very little or no starch accumulation was observed in the HLB-free SB mandarin midrib (**Figure 3A**), while starch accumulation in midribs of sensitive "Valencia" sweet orange (**Figures 4C, 5A,B**, and **Supplementary Figure S1E**) and SB mandarin siblings (**Figures 4H,J,L,N**, and **Supplementary Figures S1F–I**) was far greater than that in "Bearss" lemon (**Figure 4A** and **Supplementary Figure S1D**) and SB mandarin (**Figure 4B** and **Supplementary Figures S1B,C**). Spatially, these excessive starch grains in midribs are located analogously to the lamina in the epidermis and mesophyll cells (palisade and spongy parenchyma cells) (**Figure 4**). Over-accumulation of starch grains in the HLB-sensitive cultivars was also conspicuously found in phloem sieve elements and parenchyma cells, phloem ray parenchyma cells, xylem ray parenchyma cells (**Figure 5** and **Supplementary Figure S1**), pith parenchyma cells (**Supplementary Figure S2**), and even in tracheids (**Figure 4C** and **Supplementary Figure S2**). The pith which is encircled by a ring of xylem consisted of parenchyma cells (**Supplementary Figure S2A**). Pith parenchyma cells of "Valencia" sweet orange and SB siblings contained an abundance of starch grains (**Supplementary Figures S1E–I**). The starch grains were also found in "Bearss" lemon (**Supplementary Figure S1D**) and SB mandarin, but in substantially lower quantities (**Supplementary Figures S1B,C**).

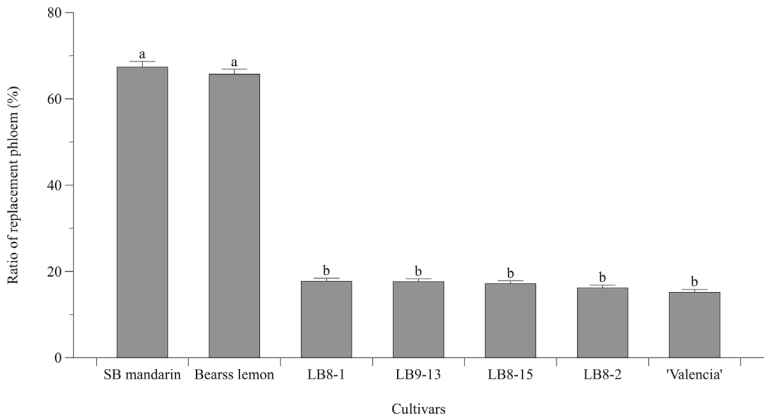


**FIGURE 6 |** Bar chart showing the ratio (%) of replacement phloem (the replacement phloem width/the total phloem width × 100). Bar and error bar denote means and 95% confidence intervals, respectively. Means that are significantly different in one way ANOVA analysis and Tukey's HSD test are represented by different small letters above bars ($p < 0.05$).

## DISCUSSION

Anatomical characteristics concerning interior structures are an integral part of the resistance of plant responses to abiotic and biotic stress, contributing to optimization of cultivation practices and selection of new and/or highly productive varieties in stress conditions (Quarrie et al., 2015). Different citrus species varied in responses to *C*Las infection (Fan et al., 2012; Fan et al., 2013). Therefore, the pathological and anatomical responses to *C*Las infection in different citrus varieties could help to identity one of the possible mechanisms underlying the HLB tolerance or sensitivity.

### Less Interior Structural Destruction and Disorganization Caused by *C*Las Infection in "Bearss" Lemon and "LB9-9" Sugar Belle® Mandarin Trees

Here, all the foliage samples from throughout the spring flushes are assumed to have been almost simultaneously subjected to severe *C*Las attack under natural Florida field conditions, within the same geographical location and under common horticultural management. Different cultivars shared some common microscopical manifestations and anatomical aberrations, but they exhibited diverse HLB-associated histopathological symptom severity.

The most notable and common disease manifestation in all the examined cultivars was abundant callose deposition. Our observations showed the increased callose deposition was mainly distributed in the new and replacement phloem area (**Figure 5**). The preference of distribution in new and/or replacement phloem area supported Achor's view that plugging is a key primary response to *C*Las infection (Achor et al., 2010) and had some resemblance to the case of American elm (*Ulmus americana* L.) with the frequent occurrence of callose deposition in replacement phloem and sieve tubes close to vascular cambial zone (Braun and Sinclair, 1976). Callose, a β-1,3 glucan, along with PP2 (phloem protein 2) of filamentous appearance, were the main obstructive media directly involved in phloem plugging, instead of the *C*Las bacterial itself, which is not found in sufficient numbers to cause phloem sieve element plugging (Kim et al., 2009; Achor et al., 2010; Fan et al., 2012; Fan et al., 2013; Johnson et al., 2014; Etxeberria and Narciso, 2015). The direct evidence is that the diameter of sieve plate pores of dicotyledons likely ranges from a fraction of 1 to 14 μm (Esau and Cheadle, 1959), while the diameter of *C*Las is from 0.1 to 0.2 μm (Bové, 2006; Wang et al., 2017). The massive callose deposition observed in all the HLB-affected cultivars here corroborated the earlier study that callose deposition plays a role as a defensive fortification of the citrus tree (Kim et al., 2009; Achor et al., 2010) to narrow the connecting strands or seal the sieve pores completely when the flow through sieve tubes becomes detrimental (Esau and Cheadle, 1959).

Another histopathological disturbance caused by *C*Las infection is characterized as phloem necrosis. Although the presence of *C*Las in the phloem was not confirmed, localized phloem necrosis was observed to scatter through vascular tissue of sweet orange leaves (Schneider, 1967; Schneider, 1968). Schneider also stated that occasionally, excessive hypertrophy of ray and phloem parenchyma cells, as well as increased differentiation of vascular cambium, and aggravated phloem tissue necrosis (Schneider, 1967; Schneider, 1968). Reportedly, the phloem necrosis could be associated with an abnormal swelling of the middle lamella between cell walls surrounding sieve elements (Folimonova and Achor, 2010) and misshapen phloem parenchyma cells (Folimonova and Achor, 2010; Brodersen et al., 2014). Here, the amount of phloem necrosis and parenchyma cell hypertrophy in "Valencia" sweet orange and SB siblings were significantly greater than that in "Bearss" lemon and SB mandarin. Further, the necrosis was widely distributed in phloem of "Valencia" sweet orange and the SB siblings, while minimal and infrequent phloem necrosis was found in "Bearss" lemon and SB (**Figure 4**).

In addition, although phloem collapse with cell wall distortion and thickening as well as cambium degeneration was observed in HLB-affected "Bearss" lemon and SB mandarin leaves, it

was much less severe than in "Valencia" sweet orange or the sensitive SB siblings. Partially or entirely collapsed sieve elements and companion cells, even in the newly generated replacement phloem and occasionally disorganized vascular cambium cells, could be easily found in the HLB-sensitive types (**Figure 4**). The significantly collapsed sieve element and companion cells showed disordered cellular organization with dissolved cytoplasmic structure, as well as both cytoplasmic and cell wall thickening, while phloem parenchyma cells were expanded to turgid size (**Figure 5**). According to Schneider's earlier study, phloem collapse was primarily due to hyperactive differentiation of vascular cambium and hypertrophy of parenchyma cells surrounding the necrotic phloem pocket (Schneider, 1968). Folimonova and Achor (2010) demonstrated that swelling of middle lamella between cell walls surrounding sieve elements potentially has been attributed to phloem collapse and necrosis.

The interior structural destruction and disorganization may contribute to an inhibition of source-to-sink transportation of photosynthetic products, as well as carbohydrate metabolism imbalances (Koh et al., 2012; Etxeberria and Narciso, 2015). As a consequence, the carbohydrate metabolism imbalance was manifested as over-accumulation of starch in the HLB-affected lamina (**Figure 2**) and midribs (**Figures 4, 5** and **Supplementary Figures S1,S2**). The unusual excessive starch distribution presented here corroborated the previous pathological observations that not only the photosynthetic mesophyll cells, vascular parenchyma cells and sieve elements but also epidermal and pith parenchyma cells were replete with starch granules (Schneider, 1968; Etxeberria et al., 2009; Achor et al., 2010; Folimonova and Achor, 2010; Gonzalez et al., 2012). In addition to the reported intracellular distribution, our anatomical results described the occurrence and distribution of starch accumulation in xylem tracheid elements (**Supplementary Figures S1H,S2**). In this present work, starch accumulation was also detected in xylem, which may be indicative of the xylem tissue as a starch deposition site and the newly formed xylem elements as strong sinks (Drossopoulos and Niavis, 1988).

Excessive starch granule content disrupts the internal thylakoid structure of the chloroplasts, which likely explains why HLB-affected leaves have an externally visible symptom of an asymmetrical chlorosis referred to as "blotchy mottle appearance" (Bové, 2006; Achor et al., 2010; Fan et al., 2013). Here, the excessive starch accumulation was also in conformity with evidence of up-regulation of starch biosynthesis encoding genes such as ADP-glucose pyrophosphorylase, starch synthase, granule-bound starch synthase, and starch debranching enzyme (Kim et al., 2009; Aritua et al., 2013), and down-regulation of functional genes related to starch breakdown process such as *DPE*2 and *MEX*1 (Fan et al., 2010). In HLB-affected samples from "Bearss" lemon and SB mandarin, starch accumulation was much less abundant than in the sensitive types.

The results presented in this work indicate that, although physically, morphologically and pathologically similar, "Bearss" lemon and SB mandarin were much less affected anatomically and structurally by *C*Las than "Valencia" sweet orange and the SB siblings; the latter also suffered more severe phloem destruction and disorganization. Internal structural preservation of the tolerant cultivars generally was superior to the sensitive types when under *C*Las pathogen attacks.

## More Replacement Phloem Generation to Compensate for the Dysfunctional Phloem in "Bearss" Lemon and "LB8-9" Sugar Belle® Mandarin Trees

In the HLB-affected citrus samples, the vascular cambium became hyperactive to generate a wide band of replacement phloem (**Figures 3D, 4, 5**). The replacement phloem consisted of the assemblages of sieve elements, companion cells, and phloem parenchyma cells, but lacked phloem fibers, and the size of the replacement band was less than the peripheral old phloem. In the HLB-affected samples of "Bearss" lemon and SB mandarin, most of the phloem cells that were not totally damaged or collapsed by *C*Las infection were likely still functional. Before any evidence of massive phloem necrosis and its eventual collapse, there has been considerable replacement phloem regenerated from the cambial differentiation zone in these tolerant cultivars (**Figure 4**). However, for the HLB-affected samples of "Valencia" sweet orange and SB siblings, phloem degeneration could occur in the peripheral old phloem of the tissue and proceeded toward the newly generated replacement phloem, even the undifferentiated vascular cambial zone. The newly generated replacement phloem rapidly became necrotic, which led to collapse (**Figures 4, 5**).

Such an analogous situation has also been reported in elm phloem necrosis with the production of new and replacement vascular phloem tissue (Braun and Sinclair, 1976). In citrus, Schneider (1968) found some necrotic but some still functional sieve tubes within the replacement phloem in HLB-affected sweet orange, corresponding to the anatomaical observations here of newly generated replacement phloem, but necrotic and/or collapsed in HLB-sensitive types (**Figures 4, 5**). According to Albrigo et al. (2014), HLB-affected samples from both young potted and field-grown trees generated more layers of new phloem cells when compared to samples from healthy control trees. The newly produced ring of metaphloem from HLB-affected citrus seems to be healthy phloem functionally in both petioles and stems (Brodersen et al., 2014). Fan et al. (2012) also reported significantly up-regulated genes involved in cell wall biosynthesis in HLB-affected rough lemon, potentially supporting the formation of new functional phloem tissues, which potentially explains one of the tolerance mechanisms of rough lemon to HLB.

"Bearss" lemon and SB mandarin produced significantly more replacement phloem (**Figure 6**), and this newly generated replacement phloem was also less affected than in the sensitive types. These traits can be regarded as adaptive and compensative to the adverse effects of *C*Las infection, contribute to the mitigation of phloem dysfunction, and help to support and maintain phloem transport longer.

### Pathological Anatomical Mechanisms Underlying HLB Tolerance of "Bearss" Lemon and "LB8-9" Sugar Belle® Mandarin Trees

HLB-affected trees have a compromised and dysfunctional phloem system. To overcome the compromised phloem, significantly more replacement phloem in HLB-tolerant cultivars was generated. The lower starch accumulation in SB mandarin and "Bearss" lemon may be a consequence of greater development of replacement phloem. As a consequence of regenerating functional phloem, the transport of photosynthates is less obstructed, and less starch accumulation is observed in SB mandarin and "Bearss" lemon. The lower levels of phloem disruption and greater phloem regeneration are two key elements that contribute to HLB tolerance in these diverse citrus cultivars. Further support of this conclusion comes from a report by Zheng et al. (2018) and Etxeberria (unpublished data, from the Final Report of CRDF Proposal #899). They demonstrated that Strigolactone (SL) applications to HLB-affected "Hamlin" sweet orange trees successfully ameliorated the adverse effects of HLB. They observed an SL-induced vascular system re-establishment, and claimed this to be one reason for the reversal of HLB symptoms, including the disappearance of starch accumulation.

Earlier *C*Las graft inoculation experiments conducted under controlled greenhouse successfully showed the high HLB tolerance of the commercial lemon cultivars, based on the minimal symptoms and continuous growth (Folimonova et al., 2009), which may be extrapolated to field performance. Similarly, in Florida citrus plantings, HLB-affected lemon or lime-like phenotypes performed better than many other citrus, with little leaf loss and the densest canopies to sustain greater growth (Ramadugu et al., 2016; Miles et al., 2017).

According to the experience of Florida citrus growers, SB mandarin and "Bearss" lemon trees, even though displaying obvious blotchy mottle symptoms on older leaves later in the season, still maintained productivity and kept quite vigorous with full canopies, when the trees were maintained with good canopy and crop load management, and proper water and nutrient management (Gmitter, 2017). Previous continued and substantial field evaluations have also shown that SB mandarin trees can endure and thrive despite *C*Las infection and typical HLB symptoms (Stover et al., 2016). Recent reports of volatile and nonvolatile metabolomics associated the possible HLB tolerance of SB mandarin with several compounds contributing to anti-microbial activity, to withstand pathogen attack (Killiny et al., 2017). From the gene expression level, there are a large number of differentially expressed genes comparing SB with the more sensitive "Clementine" mandarin, among which the most enriched GO term is secondary cell wall biogenesis (Deng et al., unpublished data), which also well supports the conclusions of this anatomical study.

## CONCLUSION

This study presents an abundance of evidence of decreased phloem destruction, together with more replacement phloem generation, that underlies the greater HLB-tolerance of "Bearss" lemon and LB8-9 Sugar Belle® mandarin, in field grown trees naturally exposed to HLB. These cultivars represent genetically diverse citrus species, but the anatomical responses associated with tolerance are the same. Together with previous reports, these new detailed observations provide more evidence of an anatomical basis for some aspects of HLB tolerance, which will hopefully ultimately lead to some new approaches to combat HLB in the near future.

## AUTHOR CONTRIBUTIONS

FG conceived and designed the study and revised the manuscript. HD performed the experiment and wrote the manuscript. DA, EE, DS, and QY helped to revise the manuscript. DA also contributed the staining methods. DS provided technical guidance using microscope and protocol. EE also provided helpful discussions, aided with interpretation of results and helped to arrange the structure of the manuscript to make it a more solid conclusion. GL, DD, and QY helped to conduct data analysis. All authors approved the final manuscript.

## FUNDING

The work was partially supported by grants from the Minute Maid (#00045972), and the China Scholarship Council (CSC, #201606990048) to HD.

## ACKNOWLEDGMENTS

We thank Misty Holt and Fabieli Irizarry for assistance with plant materials and laboratory activities.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00277/full#supplementary-material

**SUPPLEMENTARY FIGURE S1 |** Starch grains in pith parenchyma cells of midrib tisssue. **(A)** HLB-free SB mandarin, showing no starch accumulation; **(B,C)** SB mandarin; **(D)** 'Bearss' lemon; **(E)** 'Valencia' sweet orange; **(F)** LB8-1 mandarin; **(G)** LB8-2 mandarin; **(H)** LB8-15 mandarin (Starch grains in the vessels are visible); **(I)** LB9-13 mandarin. The pores in pith parenchyma cells are plasma membrane-lined pores that are responsible for spanning the adjoining cell walls to permit intercellular passage of molecules and signals.

**SUPPLEMENTARY FIGURE S2 |** Epifluorescence photomicrographs of transverse sections of LB8-2 mandarin showing the high accumulation of starch spatially located in phloem sieve elements and parenchyma cells **(A,B)**, xylem ray parenchyma cells (**B**, the red arrow indicated), tracheids elements and vessel elements (**B**, the red rectangle frame indicated), as well as the pith parenchyma cells. Scale bars = 50 μm.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 18 December 2019
doi: 10.3389/fpls.2019.01598

# Citrus Production Under Screen as a Strategy to Protect Grapefruit Trees From Huanglongbing Disease

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OPEN ACCESS

**Edited by:**
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**Specialty section:**
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

**Received:** 31 July 2019
**Accepted:** 14 November 2019
**Published:** 18 December 2019

**Citation:**
Ferrarezi RS, Qureshi JA, Wright AL, Ritenour MA and Macan NPF (2019) Citrus Production Under Screen as a Strategy to Protect Grapefruit Trees From Huanglongbing Disease. Front. Plant Sci. 10:1598. doi: 10.3389/fpls.2019.01598

Citrus production under enclosed structures can exclude the Asian citrus psyllid (ACP, *Diaphorina citri*) and eliminate the negative effects of citrus greening or huanglongbing (HLB) disease caused by *Candidatus* Liberibacter asiaticus to the grapefruit (*Citrus paradisi*) fresh fruit industry. Physically impeding the insect vector from accessing trees is a logical method to have disease-free groves. Our objectives were to assess the ability of enclosed screenhouses to exclude the ACP, stop HLB inoculation and dissemination, and improve fruit yield of in-ground and container-grown 6-year-old "Ray Ruby" grapefruit at super-high planting densities relative to open-air trees. We built a large structure to allow commercial-scale trials and tested two production systems (screenhouse and open-air), two planting systems (in-ground and potted), and two rootstocks ("Sour Orange" [*Citrus* × *aurantium*] and "US-897" [*Citrus reticulata* × *Poncirus trifoliate*]). The experimental design was a randomized complete block design split-split-plot with four replications. Four passively ventilated 1,080-$m^2$ completely enclosed screenhouses were constructed using a 50-mesh monofilament high-density polyethylene screen. The main support for each enclosed, covered structure consisted of pressure-treated, wooden utility poles. Trees were planted in Sept/2013 on a density of 1,957 trees/ha. Irrigation was performed on-demand using two 7.6-LPH drip emitters per tree, and fertigation was applied three times/week using 15N-2.6P-22.4K water-soluble fertilizer at 180 kg N/ha. Psyllids were monitored using sticky cards and detected inside the screenhouses post-Hurricane Irma, which damaged the screen structures in Sept/2017, leaving openings until repairs were completed in Apr/2018. Screen aging and a tropical storm in April/2019 caused another major screen opening fixed in Oct/2019. Despite the weather-related damages to the screens, only trees cultivated in open-air tested positive for *Candidatus* Liberibacter asiaticus after 6 years. There was fast disease progression for all outside treatments, with 100% infection. Covered, in-ground trees exhibited the highest trunk diameter and canopy volume ($P < 0.0001$). Trees grown inside screenhouses exhibited higher fruit yield than outside trees, with the highest yield observed for in-ground trees on "US-897" (51,081 kg/ha) ($P < 0.0001$). Several open-air treatments particularly in containers did not produce any fruit. On the other hand, potted grapefruit trees

cultivated inside the enclosures had the highest soluble solids content ($P < 0.001$). The screenhouses provided disease exclusion, increased fruit yield, and fruit quality, representing an alternative for growers interested in producing high-quality fruit for the fresh market. Production cost and economic viability still need to be evaluated for large-scale implementation.

**Keywords: enclosure, potted tree production, high-density, fertigation, vector exclusion**

## INTRODUCTION

Citrus is directly linked with the "Sunshine State" agricultural identity. Unfortunately, Florida's total citrus production declined 83% from 291.8 million boxes in 2003/04 to 49.58 million boxes in 2017/18, indicating a severe crisis in the fruit market (U.S Department of Agriculture, 2019). Citrus bearing acreage also declined drastically from 679,000 acres in 2003/04 to 400,900 acres in 2017/18. On-tree citrus values dropped 47% from $1.046B in 2008/09 to $0.551B in 2017/18 (U.S Department of Agriculture, 2019). Florida grapefruit production for the fresh market experienced an even steeper decline, dropping by 90% from 1.738 million tons in 2003/04 to 0.165 million tons in 2017/18. Grapefruit (*Citrus paradisi*) bearing acreage also reduced from 82,300 acres in 2003/04 to 29,800 acres in 2017/18 (U.S Department of Agriculture, 2019).

This devastating decline was first caused by State efforts to eradicate citrus canker (*Xanthomonas axonopodis* pv. citri), followed by catastrophic hurricanes in 2004 and 2005 (Ferrarezi et al., 2019), and then by the devastating citrus greening disease or huanglongbing (HLB), associated with the phloem-plugging bacterium *Candidatus* Liberibacter asiaticus (*C*Las). Eradication efforts for citrus canker were halted in 2006, while HLB has spread throughout Florida, with more than 80% of the citrus trees currently infected by the disease.

HLB is transmitted by the Asian citrus psyllid (ACP, *Diaphorina citri*) (Bové, 2006). The vector is a small 3 to 4 mm long insect. Shoots with newly developing tender leaves are needed by the females for oviposition and nymphal development; whereas adults can survive but not reproduce over winter, on mature leaves (Qureshi and Stansly, 2009). The adults require feeding on young shoots to develop and reproduce. Insect development is temperature-dependent. Under optimal conditions psyllids can go through 10–12 generations each year in Florida (Qureshi and Stansly, 2009; Qureshi and Stansly, 2010). Nymphs go through five nymphal instars to develop to adulthood in approximately 2 weeks. *Diaphorina citri* has a high reproductive rate and a single female is capable of laying several hundred eggs. The intrinsic rate of population increase in the absence of any biotic mortality factors was estimated in the range of 125–285 (Qureshi and Stansly, 2009). Nymphs and adults cause direct damage to the plants by feeding on the young shoots and leaves. Both biological and chemical methods of control provide significant reduction in the psyllid populations (Qureshi et al., 2009; Qureshi and Stansly, 2009; Qureshi and Stansly, 2010; Qureshi et al., 2014).

The planting of HLB-free trees from commercial nurseries, early removal of infected trees, application of foliar bactericides, and vector control are the most used strategies to manage the disease in the field (Dala-Paula et al., 2019). Scouting and frequent spraying of insecticides constitute the core of mitigation programs to reduce ACP population, reducing the feeding activity and the disease transmission (Stansly et al., 2014). In Brazil, frequent and coordinated insecticide application in area-wide programs is a successful strategy to manage the ACP (Bassanezi et al., 2013). Geographical isolation and mountainous landscape also naturally reduce the ACP population. In the United States, specifically in Florida, those strategies were tested through the Citrus Health Management Areas (CHMAs) and were not successful due to the environmental conditions and extremely high psyllid population (Singerman et al., 2017).

Despite the use of aggressive vector management programs, it has been virtually impossible to reduce the spread of the HLB in Florida. Completely enclosed screenhouses can physically exclude the vector and prevent disease transmission (Ferrarezi et al., 2017b). The screen acts as a physical barrier, blocking psyllids and insects larger than 50 mesh (0.297 mm) from damaging citrus trees (Ferrarezi et al., 2017a). The system provides a shaded environment with reduced solar radiation and evapotranspiration, creating an ideal environment for vigorous plant growth, increased fruit yield and improved fruit quality (Ferrarezi et al., 2017a). An HLB-free environment allows the production of high-quality fresh fruit, which retails for a high cash price due to the current high demand and shortage in supply.

The system originated at the University of Florida's Institute of Food and Agricultural Sciences (UF/IFAS) Indian River Research and Education Center in Fort Pierce, FL and has successfully been tested at the UF/IFAS Citrus Research and Education Center in Lake Alfred, FL as well (Ferrarezi et al., 2017b). The economics of citrus under protective screens (CUPS) is being determined (Schumann and Singerman, 2016). To date, there are approximately 161 ha of commercial CUPS in Florida, and 60 ha are planned (Eduardo Pines and Steven Callaham, personal communication).

The disadvantages of growing citrus indoors are related to the increase the populations of certain pests such as citrus rust mites (*Phyllocoptruta oleivora*), snow scales (*Unaspis citri*), and thrips (*Frankliniella* spp.), and diseases like citrus greasy spot (*Mycosphaerella citri*). Screenhouse construction costs are still high at approximately $10.76 per square meter, which does not include irrigation and trees. The 40- to 50-mesh high-density polyethylene screen may need replacement every 5 years (up to $5.38 per square meter) (Schumann et al., 2017).

Previous studies reported on the effect of screenhouses on internal environmental parameters and tree physiology (Ferrarezi et al., 2017a; Ferrarezi et al., 2017b). Solar radiation and reference evapotranspiration were 22% and 23.8% lower inside the screenhouses compared to the open-air. Air temperature was greater inside the screenhouses whereas wind gusts were higher in the open-air. There was no difference in cumulative rainfall between both production systems (Ferrarezi et al., 2017a). Trees grown inside the enclosed screenhouses had greater canopy area compared to the open-air plots (Ferrarezi et al., 2017b). The larger canopy growth inside the screenhouses related to higher fruit yield and fruit quality. Schumann et al. (2018) reported yields greater than 76,218 kg/ha and with good internal quality and all met grade requirements for the fresh market (100% pack-out).

Our objectives were to assess the ability of enclosed screenhouses to exclude the ACP, stop HLB inoculation and dissemination, and improve fruit yield of in-ground and container-grown 6-year-old "Ray Ruby" grapefruit at super-high planting densities relative to open-air trees.

# MATERIAL AND METHODS

## Site Location

The study was established in Nov. 2013 at the UF/IFAS Indian River Research and Education Center in Fort Pierce, FL (lat. 27° 26'N, long. 80°26'W, 10 m elevation above sea level).

## Meteorological Variables

Two weather stations (WatchDog 2900ET; Spectrum Technologies, Aurora, IL) were installed inside two of the four screenhouses, and two stations were placed in two of the open-air control plots. All weather stations were mounted on wooden posts 2.15 m above ground. The two stations outside the screenhouses were placed 18 to 33 m away from their accompanying screenhouses to avoid any unwanted shading effects of the screenhouses on the weather stations. The straight-line distance separating the weather stations within the two individual screenhouses was 58 m, and the straight-line distance separating the weather stations in the two individual open-air plots was approximately 157 m. The weather stations recorded solar radiation, air temperature, wind gust, and rainfall every 30 min. Reference evapotranspiration ($ET_o$) was calculated daily using the Penman-Monteith equation (Allen et al., 1998) (**Figure 1**). Solar radiation and wind gust data not shown.

## Treatments and Experimental Design

We tested two production systems (screenhouse and open-air), two planting systems (in-ground and potted), and two rootstocks {"Sour Orange" [*Citrus* × *aurantium*] and "US-897" ["Cleopatra" mandarin (*Citrus reticulata*) × "Flying Dragon" trifoliate orange (*Poncirus trifoliata*)]}, with four replications arranged in a randomized complete block split-split-plot experimental design. Production system was considered as the main plot, planting system as the split-plot and rootstock as the split-split-plot.

## Citrus Trees

"Ray Ruby" grapefruit trees were purchased from licensed, certified disease-free commercial nurseries (Sawmill Citrus Nursery, Fort Meade, FL and Brite Leaf, Lake Panasoffkee, FL). Trees were planted at a density of 1,957 trees/ha with spacing of 1.68 m in-row and 3.05 m between-row. Screenhouses contained four rows with eight trees each, while open-air plots contained three rows with eight trees each (total of 896 trees/0.46 ha). The same tree density was used in all treatments.

Potted trees were planted in 37.85-L plastic containers (#10 Accelerator AP-10; Nursery Supplies, Chambersburg, PA). The plastic containers were filled with a medium consisting (v/v) of 50% clean, washed silica sand, 15% Florida peat moss, 7.5% coconut fiber, 20% cypress sawdust, and 7.5% perlite (Harrell's, Lake Placid, FL). Plastic growing containers were placed upon 103-$cm^2$ ceramic tiles to prevent tree roots from growing into the underlying native soil.

In-ground trees were planted in Pineda soil series, classified as loamy, siliceous, active hyperthermic Arenic Glossaqualfs. This is very deep, nearly leveled with natural slopes ranging from 0 to 2%, and poorly drained soil.

## Screenhouses

Four passively ventilated 1,080-$m^2$ completely enclosed screenhouses (30 m wide × 36 m long × 4.3 m tall) were constructed using a 50-mesh (0.297 mm) monofilament high-density polyethylene (HDPE) screen (Signature Supply, Lakeland, FL) (**Figure 2**). The main support for each enclosed, covered structure consisted of pressure-treated, wooden utility poles (Outdoor Living Products, Orlando, FL). Each main support

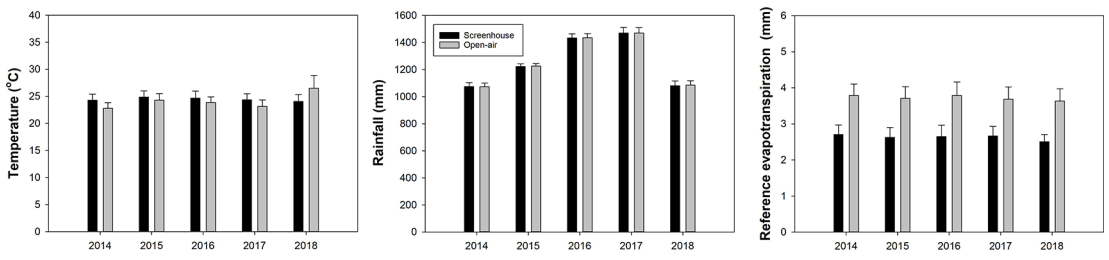


**FIGURE 1 |** Air temperature, rainfall and reference evapotranspiration inside the screenhouse and in the open-air plots. Average of 365 days per year.


**FIGURE 2 |** The University of Florida's Institute of Food and Agricultural Sciences Indian River Research and Education Center citrus under protective screen has four passively ventilated 1,080-m$^2$ completely enclosed screenhouses (30 m wide × 36 m long × 4.3 m tall) constructed using a 50-mesh monofilament high-density polyethylene (HDPE) screen (Signature Supply, Lakeland, FL). Source: Google Earth, July 2019. Credit: Rhuanito S. Ferrarezi.

utility pole was fixed to the ground with one guy-wire (1/4-inch-diameter, braided galvanized steel wire) and attached to two 5-ft-long earth anchors (Pierson Supply Company, Pierson, FL). Support utility poles located in the screenhouse corners were fixed to the ground with two guy-wires and four earth anchors (Ferrarezi et al., 2017a; Ferrarezi et al., 2017b).

The screen was attached to the sides of the structure by stapling the cloth to the interior side of the perimeter main support utility poles. The side screen cloth was attached to the top screen cloth with S-shaped galvanized steel hooks, and the side and top screen panels were pleated together, with the resulting seam directed toward the interior of the house. The construction of each screenhouse included one aluminum roll-up garage-style service door (2.44 m wide × 3.05 m tall). A 3.66-m wide × 3.66-m long × 3.66-m tall antechamber was built to limit insect inclusion when the entrance door is opened (Ferrarezi et al., 2017a; Ferrarezi et al., 2017b).

All four screenhouses and open-air plots were surrounded by a 15-m buffer area to prevent any influence on micrometeorological conditions to the next-nearest screenhouse and open-air plots.

The ground surface on both screenhouse and open-air plots were covered using a 3.66 m × 142 g weed fabric shade cloth (PRO-5 Weed-Barrier; DeWitt, Sikeston, MO) to reduce the weed population and facilitate weed control.

## Irrigation

Each tree in this trial was serviced by two 7.5-L/h flow drip emitters (SB-20; Bowsmith, Exeter, CA). Trees grown in screenhouses and open-air plots were watered to replenish the corresponding daily $ET_o$ obtained from the weather stations for their respective production systems. Monthly total and cumulative $ET_o$ values for screenhouses and open-air plots are provided in Ferrarezi et al. (2017a). From Jan. to July 2014, all trees automatically received daily irrigation volumes that were approximately 33% of the total $ET_o$ due to lower water demand. From July to Dec. 2014 onwards, trees received daily irrigation volumes that were 100% of the total $ET_o$. Trees were not irrigated on days where rainfall was equal to or greater than the $ET_o$.

## Fertigation

We used a 15N-2.6P-22.4K water-soluble fertilizer (Agrolution pHLow; Everris NA, Dublin, OH) with 15% total-nitrogen (2.6% ammoniacal and 12.4% nitrate), 2.6% phosphorus (P), 22.4% potassium (K), 3.3% calcium (Ca), 0.02% boron (B), 0.05% copper (Cu), 0.1% iron (Fe), 0.05% manganese (Mn), 0.0005% molybdenum (Mo), and 0.05% zinc (Zn). Fertilizer was mixed at a concentration of 22.68 kg fertilizer/378.5 L water in a 1,893-L plastic stock tank plumbed in-line with the servicing irrigation system. A proportional 151.4 L/min chemical injector (D8RE2; Dosatron International, Clearwater, FL) was installed directly upstream to the irrigation zone valves and connected to the fertigation stock tank. This injector added fertigation solution to each irrigation event and was adjusted seasonally to increase or decrease the proportional volume of fertigation solution added to the irrigation stream. The proportional injector's settings changed over time based on nutritional needs by season, and the minimum, maximum, and annual mean of the proportioner (v/v) were: 0.2% (February), 1.9% (September), and 0.8%. The screenhouses and the open-air plots received the same amount of fertilizer throughout the study.

## Psyllid Monitoring

Psyllids were monitored monthly from planting until April 2018, when the scouting started bimonthly. We used 10 × 17.5 cm yellow

sticky traps (Alpha Scents, West Linn, OR). The sticky cards were examined in the laboratory to search for adult psyllids.

## *Candidatus* Liberibacter Asiaticus Diagnostic

Citrus samples were screened yearly (2015-2018) or twice a year (2014 and 2019) for the presence of *C*Las. Ten fully expanded leaves and leaf petioles with intact stems from symptomatic branches (if present) were collected in eight trees per plot. Diagnostic analysis was done by the quantitative real-time polymerase chain reaction (qRT-PCR) tests coupled with U.S. Department of Agriculture's Animal and Plant Health Inspection Service approved primers (Li et al., 2006) at the HLB Diagnostic Laboratory at the UF/IFAS Southwest Florida Research and Education Center in Immokalee, FL from 2014 to 2016 and at the Southern Gardens Diagnostic Laboratory in Clewiston, FL since 2017.

## Tree Canopy Growth Parameters

Tree canopy growth parameters were measured every 6 months. Eight trees were measured per screenhouse plot for each planting system and rootstock (total $n = 64$). Six trees were measured per open-air plot for each planting system and rootstock (total $n = 48$). Tree size was assessed every year by measuring trunk diameter (5 cm above the bud union), tree height to top of canopy (not including height of vigorous shoots that extend significantly past the top of the canopy), and canopy diameter (in parallel and perpendicular to the tree row). Canopy volume was calculated using the formula: [(diameter parallel to row × diameter perpendicular to row) × height] ÷ 4.

## Fruit Yield, Fruit Diameter, and Number of Fruit

Fruit yield was determined at maturity by strip-harvesting all fruit from eight trees per plot, weighing the amount of fruit picked per tree and running it through an optical sizer (Autoline, Reedley, CA) mounted on a trailer. The number of fruit per tree and fruit diameter were determined for all fruit harvested on each tree. We categorized fruit size as small (<100 mm), adequate (100–117 mm), and large (>117 mm) based on the commercial categories used to classify grapefruit (number of fruit per cartons): size 48 and smaller, size 40–27, and size 23 and larger, respectively.

## Soluble Solids Content, Titratable Acidity, Ratio, and Yield of Solids

Random samples of 20 fruit from each experimental unit were collected for fruit quality analysis. The fruit samples were weighed, and fruit diameter at the equator was measured with a digital caliper. The fruit were cut in half, and juiced using a press juicer (model 2702; Brown International Corp, Covina, CA); then, juice was weighed, and expressed as a percentage of the total fruit weight. Soluble solids content was determined with a temperature-compensated, digital refractometer (HI96801; Hanna Instruments, Woonsocket, RI) using a few drops of juice. The total acidity (percent anhydrous citric acid) was determined by titrating juice samples to pH 8.3 with NaOH using an automatic titrator (HI931; Hanna Instruments, Smithfield, RI). The empirical soluble solids content/acid ratio, calculated by dividing the soluble solids content by the titratable acidity, is one of the most commonly used indicators of juice quality (Kimbal, 1991). Yield of solids per hectare was calculated as: (% juice in fruit ÷ 100) × (soluble solids content ÷ 100) × fruit yield (kg/ha).

## Leaf Nutritional Status

Leaf samples were collected once a year. Six leaves per tree for eight trees per screenhouse on each planting system and rootstock, and six trees per open-air on each planting system and rootstock. Healthy, asymptomatic leaves on mature, hardened-off flushes were picked, washed in phosphate-free detergent, rinsed in distilled water, and placed into a drying oven at 50°C for 5 to 7 days. Dried leaves were sent to the UF/IFAS Analytical Services Laboratories in Gainesville, FL for determination of leaf N, Mg, Mn, Zn, and Fe and Waters Agricultural Lab in Camila, GA for determination of leaf macro and micronutrients.

## Statistical Analysis

We collected six or eight sub-samples from each experimental unit depending on the production system plot size. Data were averaged to provide a single sample value representing each replication (n = 4). All variables were analyzed using general linear model procedures in SAS (version 9.4; SAS Institute, Cary, NC). The errors were checked to be normally and independently distributed, and data were presented by eight treatment combinations. Probability values were considered statistically significant when $P \leq 0.05$. Independent factor (production systems, planting systems and rootstocks) probability values are available in the **Supplementary Material (Data Sheet 1)**.

# RESULTS AND DISCUSSION

The screenhouses are able to keep psyllids out when the screen is intact. No psyllids were detected prior to 2017 according to Ferrarezi et al. (2017a). However, the subsequent passage of hurricanes or tropical storms tore the screens, allowing psyllids to enter into the screenhouses. The first single psyllid inside the UF/IFAS Indian River Research and Education Center (IRREC) CUPS was detected in May 2017, 7 months after Hurricane Matthew caused minor damages to the screen in October 2016 (**Figure 3**, top). In September 2017, Hurricane Irma caused severe damages to all four screenhouses, by loosening guy wires, bending peripheral poles, lifting off weed ground cloth, overturning all open-air pots, lifting out center poles 30 to 60 cm of ground, causing rips in screens, and provoking the detachment of S hooks. Repairs were completed in April 2018, but large openings in the screenhouse allowed psyllids to enter the enclosures. Psyllid population increased quickly and were difficult to control due to a faulty sprayer, reaching on average 15.13 psyllids in treatments in-ground and 81.25 psyllids in pots when averaging the two rootstocks. During this time, there were no significant differences among any of the treatments (**Figure 3**,

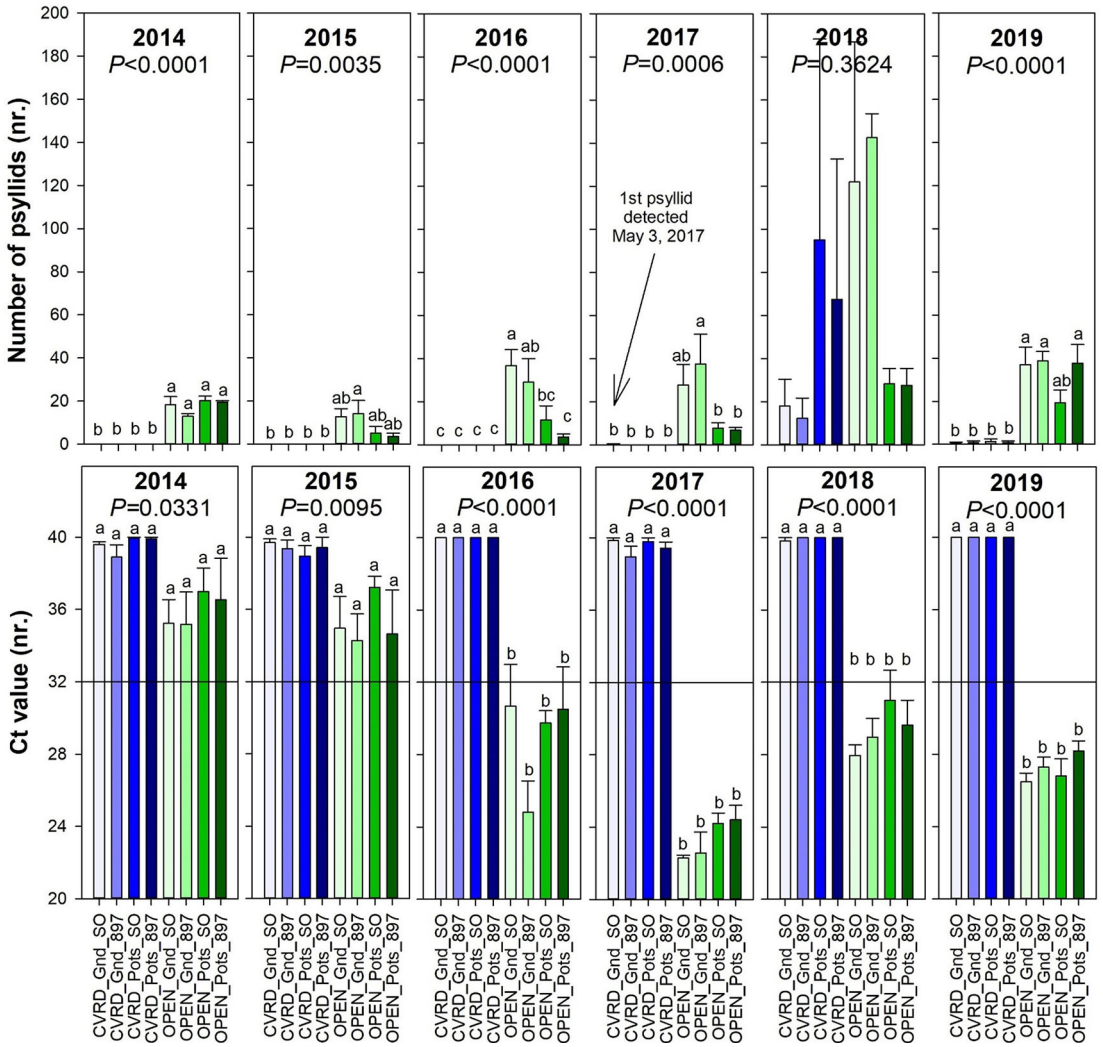


**FIGURE 3 |** Number of psyllids (top) and cycle threshold (Ct) value of *Candidatus* Liberibacter asiaticus (CLas) deoxyribonucleic acid (DNA) (bottom) of "Ray Ruby" grapefruit trees cultivated under two production systems (screenhouse, CVRD and open-air, OPEN), two planting systems (in-ground, GND and potted, POTS), and two rootstocks ("Sour Orange," SO, and "US-897"), with four replications arranged in a split-split-plot experimental design. Mean ± standard error of four replications. Means with the same letter are not significantly different from each other at 5% probability ($P \leq 0.05$) using Tukey mean comparison test. Reference line on bottom graph indicate the threshold to consider trees infected by *C*Las : < 32 negative and ≥ 32 positive.

top, $P = 0.3624$). In 2019 ≈1 psyllid was detected in in-ground trees and 1 psyllid in pots due to another tropical storm in April 2019 (**Figure 3**, top). Despite the presence of psyllids in the screenhouses in 2018 (2017 and 2019 numbers are insignificant), HLB was never detected in any of the trees. (**Figure 3**, bottom, $P = 0.0014$) It is possible the psyllids that multiplied inside the screenhouses were not infected by the *C*Las and the lack of constant re-inoculation was sufficient to prevent infection to occur (**Figure 3**, bottom, $P = 0.0014$).

Trunk diameter increased overtime in all treatments as trees continued to grow, with in-ground trees showing the highest values (**Figure 4**, top, $P < 0.0001$). There were no significant differences between the screenhouse and open-air treatments. Canopy volume increased only in the in-ground treatments (**Figure 4**, bottom, $P < 0.0001$). In 2018/19 the low canopy volume value is the result of tree hedging and topping. Potted trees resulted in the lowest volume possibly due to the growing media used: a medium consisting mainly by washed silica sand, since all the organic matter initially added was decomposed after 2 years.

Trees cultivated under screenhouses produced the greatest fruit yield. The treatment planted in-ground and on "US-897" yielded 51,081 kg/ha—twice the potted treatment on "Sour Orange" and thrice on "US-897" (**Figure 5**, $P < 0.0001$). This value is four times the 2017/18 U.S. average of 13,842 kg/ha (U.S. Department of Agriculture, 2019). Even though the fruit yield was higher than the national average, trees cultivated in pots did not perform as expected in this study. One of the justifications was the use of sand as a substrate. Over time, the organic matter decomposed, and the resulting media was 100% coarse sand. This reduced the water holding capacity and the water

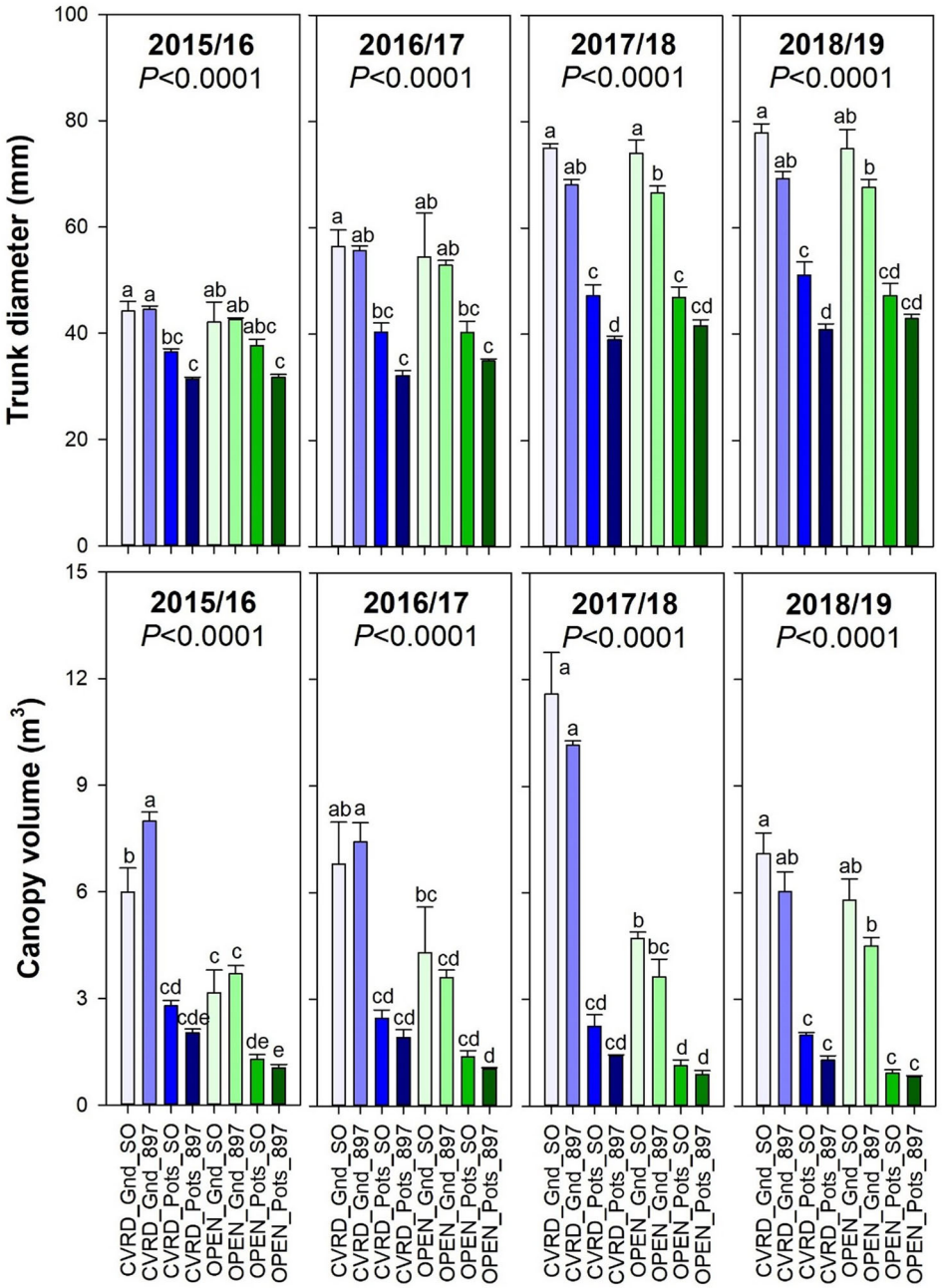


**FIGURE 4 |** Trunk diameter (top) and canopy volume (bottom) of "Ray Ruby" grapefruit trees cultivated under two production systems (screenhouse, CVRD and open-air, OPEN), two planting systems (in-ground, GND and potted, POTS), and two rootstocks ("Sour Orange," SO, and "US-897"), with four replications arranged in a split-split-plot experimental design. Mean ± standard error of four replications. Means with the same letter are not significantly different from each other at 5% probability ($P \leq 0.05$) using Tukey mean comparison test.

availability, most likely resulting in drought stresses that led to lower fruit yields. The accumulated fruit yield after 4 years was 130,306 kg/ha for trees grown under the screenhouse planted in-ground and on "US-897" followed by trees under the enclosures in pots on "Sour Orange" rootstock (**Figure 5**).

Fruit diameter and number of fruit on covered trees was statistically higher than the open-air treatments (**Figure 6**, $P < 0.0001$). The data represents the average of eight trees and four replications, and several trees in the open-air had zero fruit due to the negative effect of HLB disease. The number of fruit increased drastically in 2018/19 in comparison to previous years (**Figure 6**, bottom, $P < 0.0001$). That is expected since trees reached bearing age. However, there as a significant amount of small-sized fruit

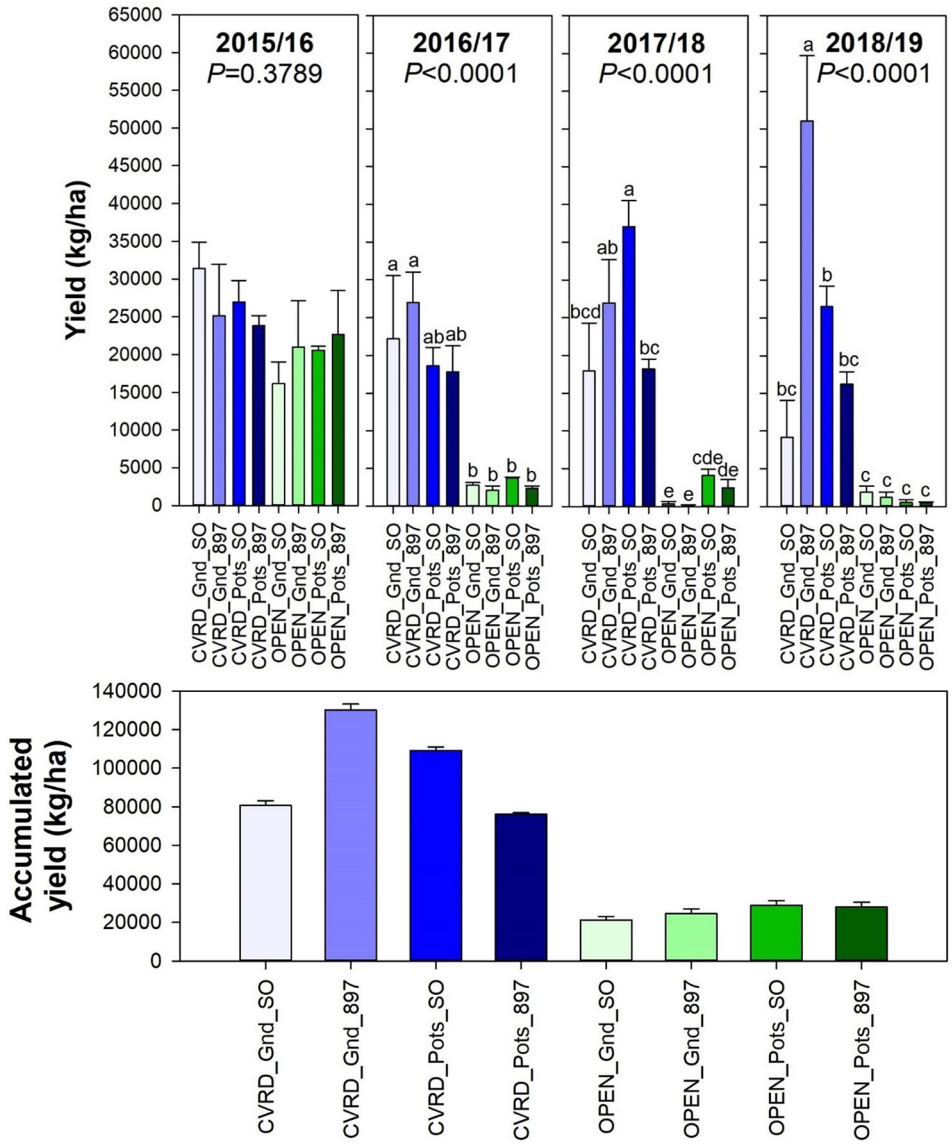


**FIGURE 5 |** Fruit yield of "Ray Ruby" grapefruit trees cultivated under two production systems (screenhouse, CVRD and open-air, OPEN), two planting systems (in-ground, GND and potted, POTS), and two rootstocks ("Sour Orange," SO, and "US-897"), with four replications arranged in a split-split-plot experimental design. Mean ± standard error of four replications. Means with the same letter are not significantly different from each other at 5% probability ($P \leq 0.05$) using Tukey mean comparison test.

(<100 mm). Fruit diameter continued to decline with progressing years in open-air treatments, likely because of the high HLB incidence.

The soluble solids content was high in potted trees with 11.8% in "Sour Orange" rootstock and 10.5% in "US-897" probably due to the constant stress induced by the growing media and limited container volume (**Figure 7**, top, $P < 0.0001$). In-ground trees in open-air resulted in the lowest soluble solids content and titratable acidity (**Figure 7**, bottom, $P < 0.0117$).

In Florida, the grapefruit harvested for the fresh fruit market needs a ratio of at least 8:1 or 8. This value changes with titrabable acidity and time of the year, and is just an industry reference. The average ratio in the 2018/19 season was approximately 7.7 (**Figure 8**, top, $P < 0.03$). Ratio declined over time, but acids tended to increase. Yield of solids represents the amount of juice per box of citrus by the soluble solids content, indicating the economic return to growers. If all yield for fresh fruit production is converted into yield of solids per hectare (not a common practice in the fresh fruit market; used for reference here), the highest amount was obtained in covered trees planted in-ground on "US-897" (1,826 kg solids/ha) and in pots on "Sour Orange" (1,419 kg solids/ha) (**Figure 8**, bottom, $P < 0.0001$).

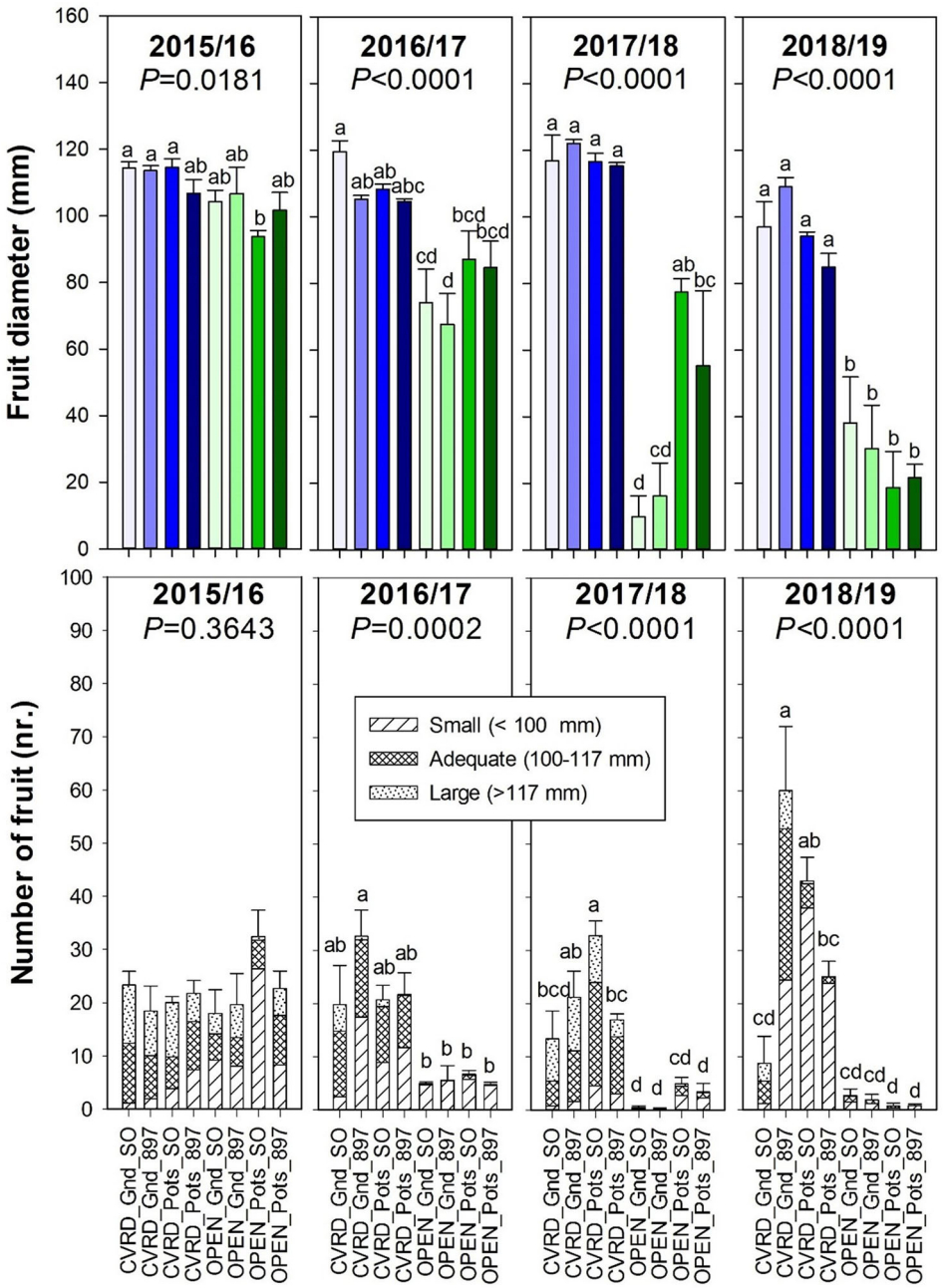


**FIGURE 6 |** Fruit diameter (top) and number of fruit (bottom) of "Ray Ruby" grapefruit trees cultivated under two production systems (screenhouse, CVRD and open-air, OPEN), two planting systems (in-ground, GND and potted, POTS), and two rootstocks ("Sour Orange," SO, and "US-897"), with four replications arranged in a split-split-plot experimental design. Mean ± standard error of four replications. Means with the same letter are not significantly different from each other at 5% probability ($P \leq 0.05$) using Tukey mean comparison test.

Nutrient content in leaf tissue varied by year as the trees grew larger (**Tables 1** and **2**, $P < 0.05$). Soon after planting from 2014 through 2016, treatment differences in leaf nutrient concentrations were observed as a result of in-ground *vs.* container-grown, and screenhouse *vs.* open-air treatments. However, no treatment effects of leaf nutrient concentrations were observed over time as tree developed. Throughout the experiment, both rootstocks exhibited similar leaf macronutrient and micronutrient concentrations. In the early stages of the experiment (2014 through 2016), container-grown trees tended to exhibit higher leaf N, P, and K concentrations than in-ground trees, but by 2017 these macronutrients concentrations equilibrated between treatments (**Table 1**, $P < 0.05$). However, this result did not extend to the micronutrient

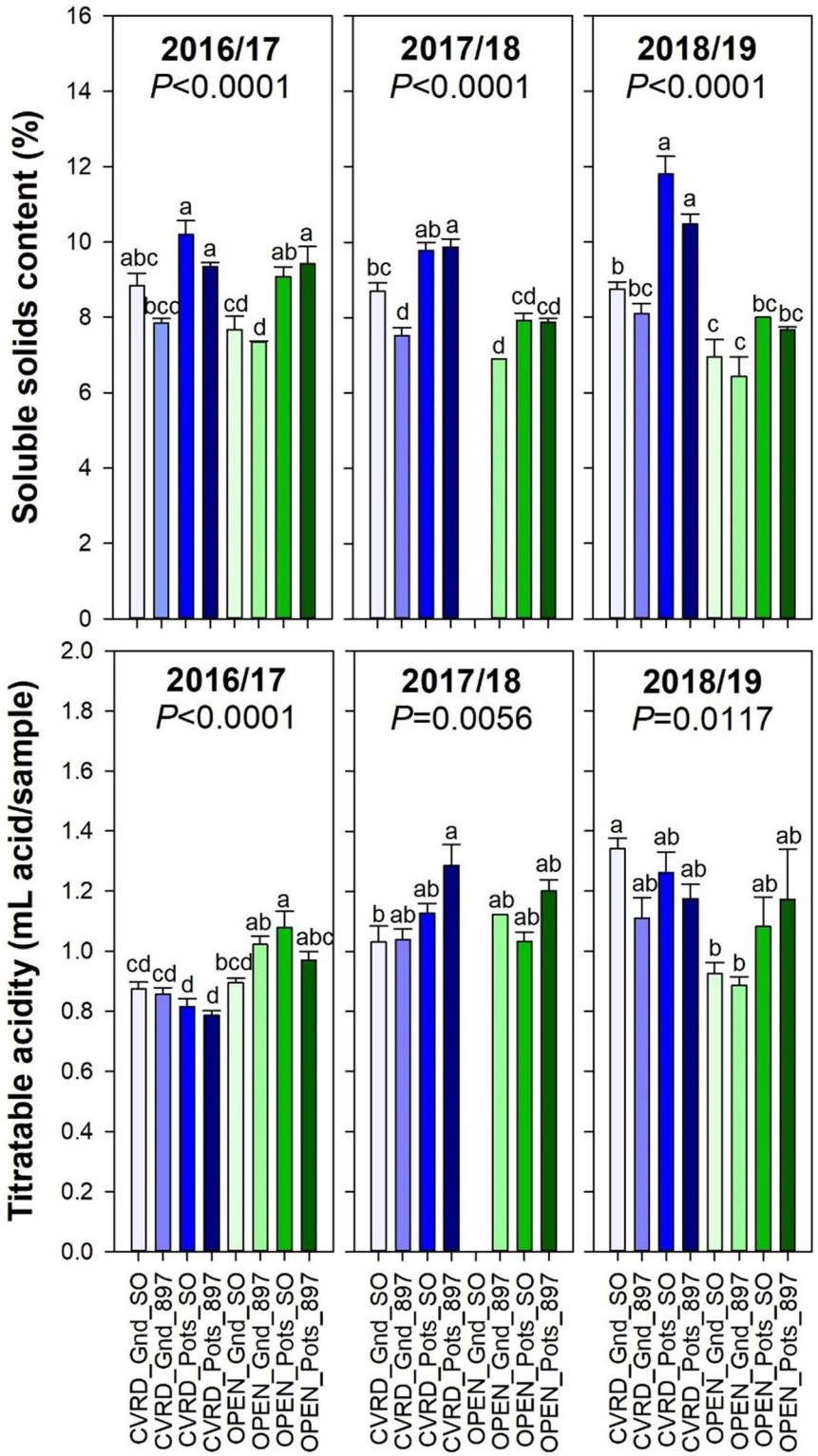


**FIGURE 7 |** Soluble solids content (top) and titratable acidity (bottom) of "Ray Ruby" grapefruit trees cultivated under two production systems (screenhouse, CVRD and open-air, OPEN), two planting systems (in-ground, GND and potted, POTS), and two rootstocks ("Sour Orange," SO, and "US-897"), with four replications arranged in a split-split-plot experimental design. Mean ± standard error of four replications. Means with the same letter are not significantly different from each other at 5% probability ($P \leq 0.05$) using Tukey mean comparison test.

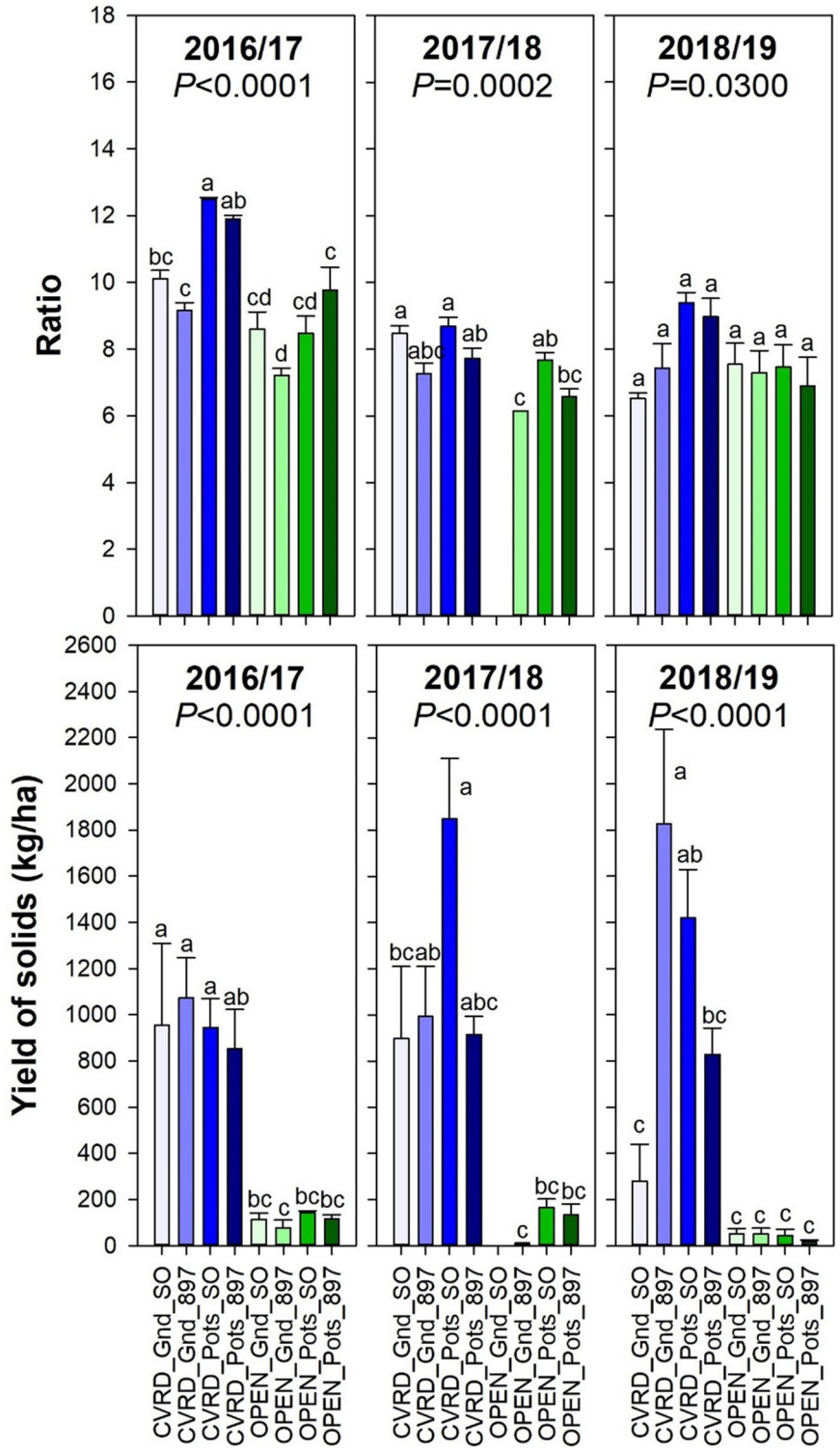


**FIGURE 8 |** Soluble solids content: titratable acidity ratio (top) and Yield of solids (bottom) of "Ray Ruby" grapefruit trees cultivated under two production systems (screenhouse, CVRD and open-air, OPEN), two planting systems (in-ground, GND and potted, POTS), and two rootstocks ("Sour Orange," SO, and "US-897"), with four replications arranged in a split-split-plot experimental design. Mean ± standard error of four replications. Means with the same letter are not significantly different from each other at 5% probability ($P \leq 0.05$) using Tukey mean comparison test. Empty bar on OPEN_Gnd_SO treatment in 2017/18 is related to the absence of fruit for fruit quality sampling in all reps.

**TABLE 1 |** Leaf macronutrient content of "Ray Ruby" grapefruit trees cultivated under two production systems (screenhouse and open-air), two planting systems (in-ground and potted), and two rootstocks ("Sour Orange," SO, and "US-897"), with four replications arranged in a split-split-plot experimental design. Mean ± standard error of four replications.

| | N (%) | | | | | | | | P (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Year** | **Screenhouse** | | | | **Open-air** | | | | **Screenhouse** | | | | **Open-air** | | | |
| | **In-ground** | | **Potted** | | **In-ground** | | **Potted** | | **In-ground** | | **Potted** | | **In-ground** | | **Potted** | |
| | **SO** | **US-897** | **SO** | **US-897** | **SO** | **US-897** | **SO** | **US-897** | **SO** | **US-897** | **SO** | **US-897** | **SO** | **US-897** | **SO** | **US-897** |
| **2014** | 2.3 c | 2.3 c | 2.8 b | 2.8 b | 2.7 b | 2.7 b | 3.2 a | 3.1 a | 0.1 d | 0.1 cd | 0.2 bc | 0.1 bcd | 0.2 abc | 0.2 ab | 0.2 a | 0.2 a |
| **2015** | 2.7 d | 2.6 d | 3.5 bc | 3.9 a | 2.9 d | 2.8 d | 3.4 c | 3.8 ab | 0.2 c | 0.2 c | 0.2 a | 0.2 a | 0.2 c | 0.2 bc | 0.2 ab | 0.2 ab |
| **2016** | 2.3 a | 2.4 a | 2.4 a | 2.4 a | 2.3 a | 2.5 a | 2.6 a | 2.6 a | 0.2 abc | 0.3 a | 0.2 bc | 0.2 c | 0.2 bc | 0.2 ab | 0.2 bc | 0.2 bc |
| **2017** | 3.0 c | 3.0 c | 3.1 bc | 3.1 bc | 3.1 bc | 3.2 abc | 3.5 ab | 3.6 a | 0.2 a | 0.2 a | 0.2 a | 0.2 a | 0.2 a | 0.2 a | 0.2 a | 0.2 a |
| **2018** | 1.9 c | 2.1 bc | 2.1 abc | 2.1 bc | 2.2 abc | 2.5 abc | 2.7 a | 2.6 ab | 0.2 a | 0.2 a | 0.2 a | 0.2 a | 0.2 a | 0.2 a | 0.2 a | 0.2 a |

| | K (%) | | | | | | | | Ca (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Year** | **Screenhouse** | | | | **Open-air** | | | | **Screenhouse** | | | | **Open-air** | | | |
| | **In-ground** | | **Potted** | | **In-ground** | | **Potted** | | **In-ground** | | **Potted** | | **In-ground** | | **Potted** | |
| | **SO** | **US-897** | **SO** | **US-897** | **SO** | **US-897** | **SO** | **US-897** | **SO** | **US-897** | **SO** | **US-897** | **SO** | **US-897** | **SO** | **US-897** |
| **2014** | 2.5 c | 2.2 c | 3.9 a | 3.8 ab | 2.5 c | 2.2 c | 3.5 ab | 3.3 b | 3.0 bc | 3.8 a | 1.9 d | 2.0 d | 2.9 c | 3.6 ab | 1.9 d | 2.5 cd |
| **2015** | 3.2 c | 2.4 d | 4.5 a | 4.2 a | 2.5 d | 2.1 d | 3.7 b | 3.6 bc | 2.6 bc | 3.6 a | 1.9 d | 2.2 cd | 2.3 cd | 3.1 ab | 2.0 d | 2.3 cd |
| **2016** | 2.3 a | 2.3 a | 2.0 ab | 1.8 bc | 1.6 cd | 1.4 d | 1.6 cd | 1.6 bcd | 4.2 abc | 4.5 abc | 4.5 ab | 4.8 a | 4.4 abc | 4.0 bc | 3.8 c | 4.3 abc |
| **2017** | 2.9 ab | 2.6 ab | 3.0 a | 3.3 a | 2.7 ab | 2.3 b | 2.7 ab | 2.9 ab | 3.6 abc | 4.1 a | 3.8 cb | 3.8 ab | 2.8 c | 2.9 bc | 2.7 c | 2.7 c |
| **2018** | 2.3 a | 2.2 a | 1.8 a | 1.9 a | 1.8 a | 1.7 a | 1.9 a | 1.8 a | 4.7 ab | 5.0 ab | 5.4 a | 5.2 ab | 4.6 ab | 4.3 b | 4.2 b | 4.2 b |

| | Mg (%) | | | | | | | | S (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Year** | **Screenhouse** | | | | **Open-air** | | | | **Screenhouse** | | | | **Open-air** | | | |
| | **In-ground** | | **Potted** | | **In-ground** | | **Potted** | | **In-ground** | | **Potted** | | **In-ground** | | **Potted** | |
| | **SO** | **US-897** | **SO** | **US-897** | **SO** | **US-897** | **SO** | **US-897** | **SO** | **US-897** | **SO** | **US-897** | **SO** | **US-897** | **SO** | **US-897** |
| **2014** | 0.2 abc | 0.2 bcd | 0.1 cd | 0.1 d | 0.2 ab | 0.2 a | 0.2 bcd | 0.2 bcd | 0.3 a | 0.3 a | 0.3 a | 0.3 a | 0.3 a | 0.3 a | 0.3 a | 0.3 a |
| **2015** | 0.1 b | 0.1 ab | 0.1 c | 0.1 c | 0.2 ab | 0.2 a | 0.1 c | 0.1 c | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| **2016** | 0.3 ab | 0.3 a | 0.3 ab | 0.3 a | 0.2 bc | 0.3 abc | 0.2 c | 0.2 bc | 0.2 ab | 0.3 a | 0.2 ab | 0.2 b | 0.2 ab | 0.2 ab | 0.2 b | 0.2 ab |
| **2017** | 0.3 ab | 0.3 a | 0.3 abc | 0.3 a | 0.2 bc | 0.2 abc | 0.2 c | 0.2 c | 0.3 ab | 0.3 ab | 0.3 ab | 0.3 a | 0.3 b | 0.3 b | 0.3 ab | 0.3 ab |
| **2018** | 0.3 a | 0.3 abc | 0.2 ab | 0.3 abc | 0.2 bc | 0.2 c | 0.2 c | 0.2 c | 0.3 a | 0.3 a | 0.3 ab | 0.3 a | 0.3 ab | 0.3 ab | 0.3 ab | 0.3 ab |

*Means with the same letter are not significantly different from each other at 5% probability ($P \leq 0.05$) using Tukey mean comparison test.*

**TABLE 2 |** Leaf micronutrient content of "Ray Ruby" grapefruit trees cultivated under two production systems (screenhouse and open-air), two planting systems (in-ground and potted), and two rootstocks ("Sour Orange," SO, and "US-897"), with four replications arranged in a split-split-plot experimental design.

| Year | B (ppm) | | | | | | | | Cu (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Screenhouse | | | | Open-air | | | | Screenhouse | | | | Open-air | | | |
| | In-ground | | Potted | | In-ground | | Potted | | In-ground | | Potted | | In-ground | | Potted | |
| | SO | US-897 | SO | US-897 | SO | US-897 | SO | US-897 | SO | US-897 | SO | US-897 | SO | US-897 | SO | US-897 |
| **2014** | 94.4 a | 94.6 a | 84.8 a | 88.7 a | 79.4 a | 78.5 a | 75.4 a | 78.5 a | 97.4 a | 93.1 a | 76.1 a | 77.0 a | 86.0 a | 83.5 a | 86.8 a | 84.9 a |
| **2015** | 75.5 ab | 79.6 a | 72.9 ab | 84.0 a | 46.5 c | 49.8 c | 66.8 b | 65.4 b | 45.3 a | 51.4 a | 23.8 b | 26.8 b | 55.9 a | 51.7 a | 51.1 a | 45.3 a |
| **2016** | 82.7 ab | 86.4 ab | 85.3 ab | 91.5 ab | 78.4 ab | 67.1 b | 80.9 ab | 98.2 a | 39.9 bc | 30.8 c | 32.8 c | 42.0 bc | 97.6 ab | 98.1 ab | 130.3 a | 107.0 a |
| **2017** | 75.3 abc | 77.2 abc | 86.1 ab | 90.1 a | 60.6 c | 56.5 c | 65.8 bc | 70.3 abc | 134.9 a | 141.0 a | 139.9 a | 119.2 a | 82.7 a | 84.0 a | 91.9 a | 94.4 a |
| **2018** | 84.9 a | 89.3 a | 90.7 a | 97.6 a | 79.9 a | 72.0 a | 75.9 a | 81.7 a | 127.1 a | 152.0 a | 137.1 a | 131.9 a | 101.0 a | 107.6 a | 103.2 a | 114.5 a |

| Year | Fe (ppm) | | | | | | | | Mn (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Screenhouse | | | | Open-air | | | | Screenhouse | | | | Open-air | | | |
| | In-ground | | Potted | | In-ground | | Potted | | In-ground | | Potted | | In-ground | | Potted | |
| | SO | US-897 | SO | US-897 | SO | US-897 | SO | US-897 | SO | US-897 | SO | US-897 | SO | US-897 | SO | US-897 |
| **2014** | 57.1 c | 70.7 bc | 76.3 abc | 70.4 bc | 74.9 abc | 97.0 ab | 107.0 a | 82.3 abc | 99.0 a | 85.7 a | 78.0 a | 84.0 a | 104.9 a | 109.0 a | 100.9 a | 102.4 a |
| **2015** | 58.9 ab | 59.9 ab | 66.2 ab | 50.5 ab | 49.7 b | 61.8 ab | 80.5 a | 61.5 ab | 39.8 a | 36.4 a | 31.1 a | 29.8 a | 27.4 a | 32.3 a | 32.2 a | 29.3 a |
| **2016** | 58.9 ab | 73.1 a | 64.3 ab | 46.9 b | 64.2 ab | 74.6 a | 61.9 ab | 51.8 ab | 17.5 c | 20.1 bc | 16.5 c | 16.0 c | 22.8 abc | 32.0 a | 29.5 ab | 25.3 abc |
| **2017** | 86.5 a | 95.6 a | 77.8 a | 77.3 a | 70.6 a | 77.0 a | 70.6 a | 66.4 a | 20.6 a | 21.3 a | 19.2 a | 20.8 a | 17.4 a | 22.9 a | 20.1 a | 19.9 a |
| **2018** | 86.5 a | 99.8 a | 88.1 a | 82.5 a | 58.9 a | 71.6 a | 60.6 a | 49.0 a | 20.1 ab | 22.5 a | 19.3 ab | 21.7 a | 16.3 ab | 20.2 ab | 17.1 ab | 13.5 b |

| Year | Zn (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Screenhouse | | | | Open-air | | | |
| | In-ground | | Potted | | In-ground | | Potted | |
| | SO | US-897 | SO | US-897 | SO | US-897 | SO | US-897 |
| **2014** | 32.0 c | 30.2 c | 33.2 bc | 32.9 bc | 40.8 ab | 42.3 a | 44.3 a | 42.0 a |
| **2015** | 18.7 ab | 18.8 ab | 21.2 a | 16.5 b | 18.0 ab | 19.0 ab | 21.8 a | 20.8 a |
| **2016** | 26.8 cd | 23.5 d | 26.6 cd | 33.4 bcd | 38.5 abc | 37.6 abc | 44.1 ab | 49.5 a |
| **2017** | 24.2 a | 26.1 a | 26.0 a | 27.3 a | 25.7 a | 28.2 a | 28.9 a | 31.2 a |
| **2018** | 20.8 a | 19.9 a | 19.7 a | 23.5 a | 23.6 a | 22.9 a | 24.6 a | 25.1 a |

*Mean ± standard error of four replications. Means with the same letter are not significantly different from each other at 5% probability ($P \leq 0.05$) using Tukey mean comparison test.*

concentrations as there was no major container *vs*. in-ground effect. Overall, there was not effect of screenhouse *vs*. open-air treatments on either macronutrient or micronutrient concentrations. For the most part, micronutrient concentrations were seldom affected by any treatment (**Table 2**, $P < 0.05$).

Ongoing studies in FL had shown horticultural feasibility in producing trees indoors due to high fruit yields and pack-outs. The economics of citrus undercover production systems is still being determined (Schumann and Singerman, 2016). The higher cost of CUPS must be offset by the highest possible yield of premium quality fresh fruit with a high market price in order to be profitable. Fortunately, the price of fresh fruit, especially for tangerine varieties, has been on an upward trend in recent years, totally justifying the investment cost. Because CUPS is a relatively new citrus production system with new challenges, current guidelines are preliminary and undergoing constant refinement through research. Mites and thrips may selectively enter through the permeable screen, while some of the larger beneficial pest predators are excluded. Greasy spot and other fungal diseases also thrive in the more humid conditions of the screenhouse environment. These non-lethal but economically important pests and diseases must be adequately controlled with integrated pest management approaches customized for CUPS in order to avoid loss of fruit yield and fruit quality.

However, these potential positive indicators are irrelevant if the screenhouse structure cannot resist high wind gusts and precipitation, and if the materials and design used to build large-scale facilities are not engineered to last and endure the weather. Most of the existing experimental and commercial facilities have been designed with limited engineering criteria, and the future of commercial CUPS in Florida lies on finding the adequate structure to resist extreme weather events since that is the highest percentage of the system initial investment.

## CONCLUSIONS

The screenhouses drastically reduced the number of psyllids in the citrus trees and avoided HLB disease, resulting in increased fruit yield and fruit quality. However, the screen effectiveness in blocking psyllids is susceptible to extreme weather events in Florida. The cost of the technology is still under evaluation along with structural modifications needed to deal with environmental challenges such as tropical storms and hurricanes. IRREC CUPS screenhouses are 6-years old, and the screen is experiencing degradation from ultraviolet light, rainfall, and wind, resulting in screen rupture at several points in the roof, requiring replacement since the material lifespan is over.

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

## AUTHOR CONTRIBUTIONS

RF, JQ, AW, MR, and NM contributed to the data collection, analysis, writing and review of the manuscript.

## FUNDING

Funding for this research was provided by the University of Florida's Institute of Food and Agricultural Sciences (UF/IFAS) Citrus Initiative, U. S. Department of Agriculture's Florida Department of Agriculture and Consumer Services (FDACS) Specialty Crop Block Grant (SCBG) (project #00092195), U. S. Department of Agriculture's Specialty Crop Research Initiative (SCRI) Citrus Disease Research and Extension Program (CDRE) (Award #2018-70016-27387), and UF/IFAS IRREC's Center Director Dr. Ronald Cave.

## ACKNOWLEDGMENTS

We thank Tom James, Randy Burton, Donald Davis, Cristina Gil, Clarence King, Dr. Barrett Gruber, Dr. Arnold Schumann, Dr. Ozgur Batuman, Dr. Mike Irey, Judy Gersony, Jerry Britt, Dr. Kayla Thomason, Shamika Finkley-Hines, Daren Cole, Cassie Kirkland, Diego Ramirez, Emily Duren, Randy Jones and Ricardo Lesmes-Vesga for their technical assistance. We also thank Sungro Horticulture (Timothy "Teo" Allen and David Wadsworth), ICL Specialty Fertilizers (Ward Gunter and Grant Cloughley), Hunter Industries (John Hemphill), Plant Food Systems (Clayton Waterhouse) and Harrell's Fertilizers (Trey Whitehurst, Erik Kellim and Matt Shook) for product donations.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01598/full#supplementary-material.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 17 January 2020
doi: 10.3389/fpls.2019.01687

# The Detection and Surveillance of Asian Citrus Psyllid (*Diaphorina citri*)—Associated Viruses in Florida Citrus Groves

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**Edited by:**
*Rhuanito Soranz Ferrarezi, University of Florida, United States*

**Reviewed by:**
*Antonio Figueira, University of São Paulo, Brazil*
*Baozhong Meng, University of Guelph, Canada*

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**Specialty section:**
*This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Plant Science*

**Received:** *06 April 2019*
**Accepted:** *29 November 2019*
**Published:** *17 January 2020*

**Citation:**
*Britt K, Gebben S, Levy A, Al Rwahnih M and Batuman O (2020) The Detection and Surveillance of Asian Citrus Psyllid (Diaphorina citri)—Associated Viruses in Florida Citrus Groves. Front. Plant Sci. 10:1687. doi: 10.3389/fpls.2019.01687*

The plant pathogenic bacterium *Candidatus* Liberibacter asiaticus (CLas), the causal agent of the citrus disease Huanglongbing (HLB), and its insect vector, the Asian citrus psyllid (ACP; *Diaphorina citri*), have been devastating the Florida citrus industry. To restore the competitive production presence of Florida in the worldwide citrus market, effective and sustainable control of HLB and the ACP needs to be identified. As alternatives for resistance-inducing insecticides, viruses are currently being considered for biological control of the ACP. To identify possible biological control candidates, we conducted one of the most comprehensive surveys of natural ACP populations in major citrus production regions spanning 21 counties in Florida. By optimizing PCRs and RT-PCRs, we were able to successfully detect and monitor the prevalence of five previously identified ACP-associated RNA and DNA viruses throughout Florida citrus groves, which include: *Diaphorina citri-associated C virus* (DcACV), *Diaphorina citri flavi-like virus* (DcFLV), *Diaphorina citri densovirus* (DcDNV), *Diaphorina citri reovirus* (DcRV), and *Diaphorina citri picorna-like virus* (DcPLV). Adult and nymph ACP populations from 21 of Florida's major citrus-producing counties were collected each month during approximately 18 consecutive months. RNA extracts used for these viral screens were also regionally combined and subjected to High Throughput Sequencing (HTS) to reveal a more comprehensive picture of known and unknown viruses in Florida ACP populations. We discovered that DcACV was the most prevalent ACP-associated virus throughout nymph and adult ACP populations in Florida, detected in more than 60% of all samples tested, followed by DcPLV and DcFLV. HTS allowed us to identify a novel ACP-associated reo-like virus and a picorna-like virus. The putative reo-like virus, tentatively named *Diaphorina citri* cimodo-like virus, was later surveyed and detected back in seasonal adult and nymph ACP samples collected in Florida during this study. HTS generated data also revealed that the most abundant virus in Florida ACP populations was *Citrus tristeza virus* (CTV), which is not an ACP-associated virus, suggesting persistent presence of CTV infection in citrus throughout Florida groves. Collectively, information obtained from our study may be able

to help guide the direction of biotechnological pest control efforts involving a number of viruses that were detected for the first time in Florida ACP populations, including two newly identified ACP-associated viruses.

**Keywords: Asian citrus psyllid, insect viruses, Candidatus Liberibacter asiaticus, Huanglongbing, biological control**

## INTRODUCTION

The bacterial pathogen, *Candidatus* Liberibacter asiaticus (*C*Las), the causal agent of the citrus disease Huanglongbing (HLB), continues to endanger the worldwide citrus industry, with the most severe losses occurring in Florida (Jagoueix et al., 1994; Garnier and Bové, 1996; do Carmo Teixeira et al., 2005). Currently, this disease threatens the future of Florida's $9 billion citrus industry and simultaneously threatens production sites in Texas and California (Gottwald, 2010; Hall et al., 2013). The fastidious, phloem limited *C*Las bacterium cannot be cultured, preventing the completion of Koch's postulates and *in vitro* biological studies (Jagoueix et al., 1996; do Carmo Teixeira et al., 2005). Although short-term disease management using bactericides, plant immune activators, nutritional supplements, and heat treatments have been able to slightly reduce the acceleration of the citrus plant decline, the HLB pathogen shows vigorous strength against them and eventually overcomes these temporary therapies (Li et al., 2016; Blaustein et al., 2018; Munir et al., 2018).

The insect vector of this bacterium, the Asian citrus psyllid (ACP; *Diaphorina citri* Kuwayama), has been proven to be the primary means of dispersal of this disease throughout citrus groves in Florida (Hall et al., 2013). It has also been shown that each feeding event contributes substantially to the development of disease severity and on the ultimate survival of the infected trees (Stansly et al., 2014; Lee et al., 2015). ACP control is therefore essential in Florida citrus groves and the growers' investment in vector control could still have considerable effect on the production of their trees, even with *C*Las infection (Stansly et al., 2014). Current HLB disease surveys of Florida citrus groves show approximately 95% of trees are symptomatic, with no signs of decreasing incidence. Management options for HLB are limited and rely heavily on insecticides for controlling ACP populations, even when integrated with various cultural control methods (Grafton-Cardwell et al., 2013; Blaustein et al., 2018). Unfortunately, these chemical strategies are mostly ineffective due to challenges associated with repetitive and expensive applications and the inability to consistently decrease ACP populations. Growers are also concerned with the development of chemical resistance among ACP populations and the threat long-term chemical applications pose to the environment and beneficial organisms (Tiwari et al., 2011; Hall et al., 2013; Kanga et al., 2016; Chen et al., 2017; Chen and Stelinski, 2017; Pardo et al., 2018). To prevent new citrus plantings from HLB infection and to avoid the development of pesticide-resistant ACP populations, many researchers are targeting control of the vector through biological means (Chen et al., 2018; Tian et al., 2018).

As the most abundant entity on the planet and in the ACP, viruses offer limitless molecular and biotechnological opportunities to explore and can provide further knowledge of the *C*Las-vector interaction. Virus-induced gene silencing (VIGS), which has been documented as an effective way to deliver RNA interference (RNAi) into both plants and insects, is a molecular strategy for the biological control of the ACP and eventual disruption of the HLB disease cycle (Baulcombe, 1999; Hall et al., 2013; Nandety et al., 2015; Joga et al., 2016; Galdeano et al., 2017). Another phloem-limited citrus pathogen, *Citrus tristeza virus* (CTV), is currently being used as a viral vector to deliver this RNAi technology into the citrus phloem and then into the ACPs that feed on citrus (Hajeri et al., 2014; Killiny et al., 2019). However, these routes can be cumbersome, require an established infection in the citrus plant before delivering RNAi to the insect, and may target only a few insect organs other than the gut. Employing an endogenous ACP-associated virus that may be naturally replicating and expressing its proteins in the insect can have the potential to deliver RNAi with higher efficiency and lower chances of deleterious, off-target effects.

As a viable alternative to chemical controls, development of knowledge-based and efficient biological control strategies to disrupt *C*Las transmission by current technologies (i.e., RNAi) in the ACP vector itself represent our strategy to control the disease without relying solely on chemical applications. The necessity of a precise and specific biological control for Florida ACP populations initiated this study, with hopes to provide additional molecular tools. In order to decrease or eliminate major dependence on broad-spectrum chemical controls, this study aimed to investigate the Florida ACP virome for identifying an ACP-associated virus with the potential to be manipulated into a biocontrol agent against the HLB insect vector. The endemic establishment of both HLB and the ACP in Florida has greatly increased the need for alternative control options.

Prior to this study, Nouri et al. (2016a) used metagenomic analysis in a limited number of worldwide ACP samples and identified five new viruses, with the assemblage of nearly complete viral genome sequences (Nouri et al., 2016a; Nouri et al., 2016b). These included several putative novel viruses associated with *D. citri*, such as a picorna-like virus, a reovirus, a densovirus, a bunyavirus, and an unclassified (+) ssRNA virus (Nouri et al., 2016a; Nouri et al., 2018).

The Nouri et al. (2016a) study and our initial observations of various viral particles in the ACP nymphs and adults *via* transmission electron microscopy (TEM) (unpublished data) lead us to our current strategy for identifying and detecting ACP-associated viruses, especially in Florida ACP populations. Additionally, *C*Las survival, reproduction, and dissemination has been observed to occur principally in the gut of the ACP, which

has influenced supplementary targeted approaches for potentially inhibiting bacterial persistence in this organ (Ammar et al., 2011; Ghanim et al., 2016; Ghanim et al., 2017). It was observed that apoptosis, an ACP defense response to microbial invasion, was activated to target and destroy (or limit) the *C*Las infection and spread. Ghanim et al. (2017) documented the occurrence of several upstream events leading to these apoptotic responses, which commenced in the endoplasmic reticulum (ER) of the ACP gut. In addition, the same study provided direct evidence of *C*Las replication in the midgut cells of ACP, which occurs inside Liberibacter-containing vacuoles and associates with the gut ER (Ghanim et al., 2017). Thus, an unknown virus in the gut, if manipulated, could serve as a highly efficient and selective biological control agent for ACP when consumed by the ACP and initiated our search for this virus as a viable ACP biocontrol option. It is still unknown whether these viruses are beneficial or destructive to ACPs; however, their presence in ACP populations around the world suggests the likelihood of high persistence and may therefore be a conceivable vector for RNAi delivery. Through this study, which we believe is the most comprehensive survey in natural ACP populations (i.e., not laboratory-reared) in Florida, we intend to further characterize the ACP virome as it relates to Florida citrus groves, where the HLB epidemic has infiltrated and caused unprecedented economic damage. This may bring us closer to identifying the unknown ACP-associated virus localized in the gut. We believe the future of biological control using manipulated ACP-associated viral vectors for RNAi delivery will involve targeting and blocking an essential gene in the insect to control HLB.

The main objectives of this study include surveying and monitoring previously characterized ACP-associated viruses in nymph and adult ACP populations in Florida citrus groves with PCR-based methods and simultaneously subjecting subsamples of these populations to High Throughput Sequencing (HTS) technologies. The first objective will provide foundational knowledge of the known viruses that might already be present in Florida ACP populations, with regards to their spatial and temporal characteristics throughout citrus-producing counties. The second objective will provide a more thorough analysis of the Florida ACP virome, including the detection of novel and other known viruses. By analyzing ACPs from around the world, Nouri et al. (2016a) had identified and characterized new ACP viruses from a small number of psyllid colonies; however, we've concentrated our samples in this study toward larger and naturally occurring ACP populations collected solely from Florida citrus groves and focused our efforts on discovering or identifying other robust and novel Florida ACP viruses. Results of this study may uncover a better candidate for an alternative viral vector used for delivering RNAi technology to combat the ACP in Florida.

# MATERIALS AND METHODS

## ACP Collection and Processing

Adult and nymph ACPs were collected directly from citrus trees in commercial groves in 21 of Florida's major citrus-producing counties around south-central Florida every month over a period of approximately eighteen months (August 2017 through December 2018). One grove, ranging from 500 to 2,000 acres (~200 to 800 hectare), was sampled per county and samples were taken randomly from multiple trees in one block of each grove usually around the perimeter of the block, where the observation of ACP incidence has been the greatest (Sétamou and Bartels, 2015). Each citrus grove sample site, which represented each county, remained consistent throughout the entire study, except for a few groves that closed or changed operation during the months of our sampling. These sites were immediately replaced with another grove site within the same county.

Young citrus shoots with adult or nymph ACPs were carefully detached from trees and immediately submerged into 50 ml Falcon tubes containing 100% ethanol, killing the ACPs, and the Falcon tubes were kept closed. Since HLB and ACPs are currently widespread in Florida, traveling throughout Florida with (dead) ACPs was not a concern. The number of ACPs collected varied by grove; however, we aimed to collect a minimum of 25 adults and 25 nymph ACPs from each grove every month. There were times in which lower numbers were collected due to inhospitable conditions, such as cooler or rainy weather, or increased pesticide applications. ACPs collected from these 21 groves were brought to the laboratory at the Southwest Florida Research and Education Center (SWFREC) in Immokalee FL, where adult and nymph ACPs were separated out from plant parts, counted with the aid of a stereo microscope (Leica S8 APO, Leica Microsystems, USA), and stored separately in smaller vials containing 100% ethanol at -20°C until needed. Total ACP counts per grove site (representing one county) were then calculated and combined into six regions for data analysis (**Table 1**).

## Total RNA Extraction and cDNA Synthesis

For each monthly-collected sample representing a county, a subset of five adult ACPs and five 5$^{th}$-instar nymph ACPs were randomly selected and separately used for RNA extraction. This was intended to get a snapshot of the viral content (virome) of the psyllid population in each county and each month. Preliminary tests (data not shown) and previous research together determined that five insects of this size (2–5 mm) was sufficient to yield enough total RNA for RT-PCR screenings (Runckel et al., 2011). To verify total RNA extraction and cDNA quality of ACP samples, primers were designed to target the

**TABLE 1** | Total number of ACPs collected in each Florida region over a period of 18 months.

| Regions | Number of groves/ counties | Total number of samples | Adults | Nymphs | Total |
|---|---|---|---|---|---|
| Region A | 4 | 124 | 1,933 | 14,053 | 15,986 |
| Region B | 3 | 72 | 836 | 4,535 | 5,371 |
| Region C | 5 | 150 | 1,987 | 15,187 | 17,174 |
| Region D | 4 | 115 | 884 | 8,104 | 8,988 |
| Region E | 4 | 99 | 1,578 | 13,801 | 15,379 |
| Region F | 1 | 61 | 876 | 3,651 | 4,527 |

*Diaphorina citri* (*Dc*) wingless gene as an internal control (**Table 2**). Five insects (nymphs or adults) were placed into sterile 1.5 ml Eppendorf tubes with three 2.3 mm chrome steel beads (BioSpec Products Inc, Bartlesville, OK, USA) and sealed with Breathe-Easy tube membranes (Genesee Scientific, CA, USA). All samples were then placed into a Labconco FreeZone 6 Freeze-Dry System (Kansas City, MO, USA) overnight and ground in a Mini Beadbeater™ (BioSpec Products Inc, Bartlesville, OK, USA) to a fine powder. Total RNA was extracted using the Quick-RNA MiniPrep kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions, excluding the DNase treatment in order to screen for DNA viruses in the sample. RNA was stored at -20°C until needed.

Six microliters of extracted total RNA from ACP and 1.5 µl of a random hexamer primer (250 ng/µl) (Thermo Fisher Scientific, Waltham, MA, USA) were mixed and incubated at 65°C for 5 min followed by incubation on ice for 5 min to begin complementary DNA (cDNA) synthesis. One microliter of Superscript II (200 u) reverse transcriptase (Invitrogen, Carlsbad, CA, USA), 1 µl of dNTPs (10 mM), 2 µl of DTT (0.1 mM) (Thermo Fisher Scientific, Waltham, MA, USA), 4 µl of buffer (5X) (Thermo Fisher Scientific, Waltham, MA, USA) and 4.5 µl of RNAse-free $H_2O$ were added in a total reaction mix of 20 µl and incubated at 42°C for 1 h. The cDNA was then heated to 65°C for 15 min to deactivate Superscript II. The cDNA was either used immediately in RT-PCR or stored at -20°C until needed.

## ACP-Associated Virus Screenings With RT-PCR and PCR

The cDNA and total RNA from all samples were used to screen all monthly-collected county samples for four previously identified ACP-associated RNA viruses and one DNA virus: *Diaphorina citri-associated C virus* (DcACV), *Diaphorina citri flavi-like virus* (DcFLV), *Diaphorina citri picorna-like virus* (DcPLV), *Diaphorina citri reovirus* (DcRV), and *Diaphorina citri densovirus* (DcDNV), respectively (Marutani-Hert et al., 2009; Matsumura et al., 2016; Nigg et al., 2016; Nouri et al., 2016a; Nouri et al., 2016b). These five viruses were pursued further in this study because of their previous detection in ACP populations (Marutani-Hert et al., 2009; Nouri et al., 2016a). An internal control for ACP cDNA, ACP (*Dc*) wingless gene, was also used in order to verify the integrity of the RNA for each sample. The PCR primers used for each virus, along with the target gene and the length of the amplicon, are listed in **Table 2**. All PCRs were performed using DreamTaq Green (2X) Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) in a reaction volume of 25 µl. The final concentration of each primer was 0.5 µM. The PCR cycles for each ACP-associated virus consisted of a 2-min initial denaturation at 94°C, followed by 35 cycles of 94°C for 20 s, 50°C (DcACV and DcRV), 62°C (DcDNV, DcFLV, and DcPLV), and 60°C (ACP [*Dc*] wingless gene) for 20 s and 72°C for 2 min, with final extension at 72°C for 10 min. PCR products were separated on 1% agarose gels and visualized by staining with Apex™ Safe DNA Stain (Genesee Scientific, San Diego, CA, USA). Randomly selected amplicons with expected size generated from genomic DNA (DcDNV) and cDNA (DcACV, DcFLV, DcPLV, and DcRV), and from corresponding positive control plasmids (kindly provided by Bryce Falk, UC Davis) were purified using the DNA Clean and Concentrate kit (Zymo Research, Irvine, CA, USA). The purified DNA products were sent for Sanger sequencing (MCLAB, South San Francisco, CA, USA) and generated sequences were subjected to the National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST) program to validate the target amplicons' identity.

**TABLE 2** | List of primers, gene targets, and amplicon lengths generated to detect the presence of each ACP-associated virus in all samples.

| Virus | Primers | Gene target | Length of amplicon |
|---|---|---|---|
| **DcACV** | F: 5' GCCGCACGAAACTAGTGATAAACGCA 3'<br>R: 5' GGATCGGTGGTGCACGAGTATGTAAGTA 3' | RNA1[1] segment | 473 bp |
| **DcFLV** | F: 5' AGGCGAGTACTCCCATCGGATACATT 3'<br>R: 5' GAGGGCCGCTAAGTCTGTAGGACATATT 3' | RdRp[2] | ~1.4 kb |
| **DcDNV** | F: 5' AGTCGGTGAGACTGATATCTTCGAGACC 3'<br>R: 5' GTTTAGTTCGCTTGTCGGTTACACAGG 3' | RdRp[3] | ~1 kb |
| **DcRV** | F: 5' TTTTCCCAGGTACATCGA 3'<br>R: 5' ACCATTCAGCCAGTCCTA 3' | p8[4] | 900 bp |
| **DcPLV** | F: 5' TAGGTGAACGTGATAATCCTGGTAT 3'<br>R: 5' CAGAACGTCTGTTATGAATCGGAC 3' | Polyprotein[5] | 698 bp |
| **DcCLV 5'-end** | F: 5' AACACCCATGCTTCCAAAAC 3'<br>R: 5' TCGTTTCACTAACGCCAATTT 3' | Predicted RdRp[6] | 492 bp |
| **DcCLV 3'-end** | F: 5' ATTTAGGGCCATGTGCAAAG 3'<br>R: 5' CCAACACACCGAGCATACAC 3' | Predicted RdRp[6] | 526 bp |
| **Internal control for integrity of ACP cDNA** | F: 5' TCCAGAGTGATGGTCAGTAA 3'<br>R: 5' GATCTCCTGTGTTCTGTAGC 3' | ACP (*Dc*) wingless gene[7] | 273 bp |

*[1]Accession number: KX235518.1; [2]Accession number: KX267823.1; [3]Accession number: KX165268; [4]Nouri et al, 2016a; [5]Accession number: KT698837.1; [6]PCR primers designed for novel candidate virus from high throughput sequencing (HTS) results and used for screening of ACP populations in Florida; [7]Accession number: XM_008486571.2. DcACV, Diaphorina citri associated C virus; DcFLV, Diaphorina citri flavi-like virus; DcDNV, Diaphorina citri densovirus; DcRV, Diaphorina citri reovirus; DcPLV, Diaphorina citri picorna-like virus; DcCLV, Diaphorina citri cimodo-like virus; F, Forward Primer; R, Reverse Primer; RdRp, RNA-dependent RNA polymerase; bp, base pair; kb, kilobase. An internal control was also used to verify the integrity of RNA extraction and cDNA synthesis steps.*

## Total RNA Preparation and HTS

The six larger Florida regions (Regions A–F), as depicted in **Figure 1**, were established to group all smaller county samples, which will be used in all future analyses of the study. However, based on our increased access to high numbers of ACPs in Collier County specifically, this county was separately analyzed throughout the study and referred to as Region F. Total RNAs for HTS analysis were extracted from ACP samples using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. For HTS, each regional sample consisted of pools of five whole insects (nymphs or adult ACPs) from monthly-collected county samples from early months of the study (August 2017–May 2018) that showed variation in viral incidences in the viral screening methods described above. Total RNAs extracted from each county sample were measured and verified of sufficient quality and purity for HTS using a Synergy HTX plate reader (BioTek Instruments, Winooski, VT, USA) and then combined into the six regional samples.

All six RNA samples were sent to Foundation Plant Services at UC Davis, CA, USA. Here, separate aliquots of the six samples of total RNAs were prepared and each subjected to ribosomal RNA depletion and subsequent cDNA library construction using a TruSeq Stranded Total RNA with Ribo-Zero Plant kit (Illumina, San Diego, CA). All six samples were then sequenced on the Illumina NextSeq 500 platform as previously described (Al Rwahnih et al., 2018).

## Assembly and Sequence Analysis

The resulting Illumina reads were subjected to adapter trimming and *de novo* assembled into contiguous consensus sequences (contigs) with size lengths of at least 200 base pairs by CLC Bio Genomic Workstation (v8.5.1; Qiagen, Hilden, Germany). These contigs were then annotated by a multistep process for generation of candidate viral sequences as previously described (Al Rwahnih et al., 2018). The first group of contigs was generated with the tBLASTx program (v. 2.4.0) for comparison of sample contig sequences against the viral genome database of the NCBI Refseq (https://www.ncbi.nlm.nih.gov/genome/viruses/) (Tatusova and Madden, 1999). It was determined that any contigs matching viral genomes with a combined E-value equal to or less than $10^{-4}$ were possible viral candidates.

## Verification of Novel Sequence From HTS Data in ACP Populations

Based on the assembled list of contigs compiled from HTS results, RT-PCR primers were designed to target the 5' and 3' ends of a novel viral sequence detected in HTS. The sequences of the designed primers are shown in **Table 2**. Seasonal representative samples of ACPs from the surveyed sites were then subjected to a new set of RT-PCR analysis for this viral sequence as previously described. This assay was done in order to establish preliminary prevalence of this novel virus in the regional Florida samples as well as to validate our HTS findings.

## Statistical Analysis

For statistical analysis of ACP-associated viruses, the results for the individual county-collected samples were combined into the same six Florida regions that were established for HTS analysis (**Figure 1**). JMP 14 (SAS Corp., USA) statistical software was used to analyze the significant differences between seasonal means of the prevalence of each virus between these six regional Florida samples, comparing adults and nymphs together. Means of the detected presence of each virus in each

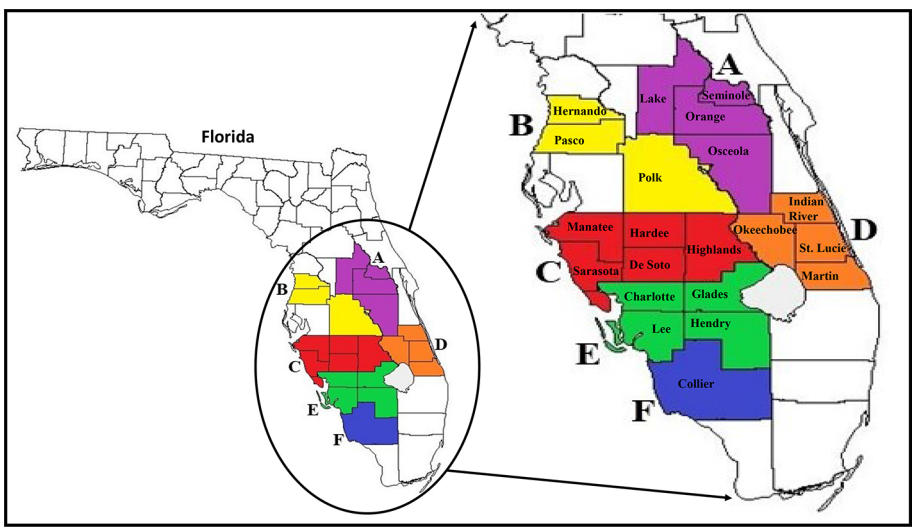


**FIGURE 1 |** Map of Florida and major citrus production areas (circled) where surveys were conducted in the state. Enlarged map on the right shows the 21 counties (coloured jigsaws with county name) where ACP populations were collected from and the six larger Florida regions (consisting of counties with the same color; **A**–**F**) established for use in all analyses. ACP, Asian citrus psyllid.

regional sample were compared to each other using the Student's t test, with significance thresholds set at $P < 0.05$.

# RESULTS

## ACPs Collected From Commercial Citrus Groves

Twenty-one groves, each representing one county in Florida's major citrus producing areas, were identified for surveys. Asian citrus psyllid (ACP) adults and nymphs from each citrus grove were collected monthly (**Figure 1**). During this study, we were able to find and collect a sufficient number of ACP adults and nymphs (n = 25–100ACPs) from each surveyed citrus grove regardless of the collection period (i.e., season). This indicates that ACP overwinter in both adult and nymph forms in Florida citrus groves. The number of ACPs collected, both adults and nymphs, was recorded separately for each grove (representing one county), but for simplicity of data presentation and statistical analyses these numbers were grouped into larger Florida regions (**Table 1** and **Figure 1**). Some monthly-collected county samples lacked collection of either nymph or adult ACP populations, most likely due to inhospitable grove conditions or environmental factors around the time of survey (i.e., recent pesticide application or rain).

## ACP-Associated Virus Screenings With RT-PCR and PCR

Based on our validated virus RT-PCR screenings of ACPs, all of the previously characterized viruses including DcACV, DcFLV, DcDNV, DcRV, and DcPLV were successfully detected in nymph and adult ACP populations collected in Florida citrus groves. Four of the five viruses (DcACV, DcPLV, DcFLV, and DcDNV) were detected in all regions sampled whereas DcRV was detected in only Region A and D (**Table 3** and **Figure 2**). Since viral prevalence showed relative consistency between seasons collected during the study (data not shown), here we present our data showing the more observable differences of viral prevalence between regions (**Figure 2**). The most prevalent virus observed in both nymph and adult ACPs was DcACV (in 67% of the samples) and was detected in every region (**Table 3** and **Figure 2**). The second most prevalent ACP-associated virus detected was the DcPLV, which was detected in 20% of the samples (**Table 3**). Although DcPLV was detected in all regions, its detection was less than DcACV and represented approximately a fifth of the viral detection in each region, except for Region F (**Figure 2**).

The DcFLV was found to exist at very close incidence levels to DcPLV (in 19% of the samples) and was detected in both adult and nymph ACPs in all regional Florida samples, though at much lower incidence levels than DcACV; however, it was found to be the third most prevalent virus (**Table 3** and **Figure 2**). The densovirus, DcDNV, was also detected in all regions, but at a much lower incidence level (7% of all samples) than DcFLV or DcPLV (**Table 3** and **Figure 2**). Finally, DcRV was the least prevalent ACP-associated virus detected in less than 1% of the samples and was found in only two of the regions during the entire study (**Table 3** and **Figure 2**).

Incidence levels of DcPLV, DcFLV, DcDNV, and DcRV remained consistently low during the extent of the survey. In contrast, incidence levels of DcACV had a higher level of variation across the regions (**Figure 2**), yet the presence of this RNA virus in ACPs between the different regions was consistently the highest ($P < 0.05$). For example, Region A (**Figure 2A**) and Region E (**Figure 2E**) show DcACV present in these areas, but at different incidence levels throughout the study. DcDNV and DcPLV that were not detected previously in Florida ACP populations (Nouri et al., 2016a) appeared to display a contradicting case during this survey and were actually widespread in Florida. However, DcDNV was found at lower incidence levels than the other viruses (**Figure 2**).

**TABLE 3** | Summary of Florida citrus grove survey results showing the total number of sites (grove/county) surveyed in each region and the number of samples in which ACP-associated viruses were detected in ACPs collected at these sites.

| Survey Regions | Total number of samples (adults and nymphs) | Number of sites (grove/ county) | Number of samples with *Diaphorina citri*-associated C virus (DcACV) | | Number of samples with *Diaphorina citri* flavi-like virus (DcFLV) | | Number of Samples with *Diaphorina citri* densovirus (DcDNV) | | Number of samples with *Diaphorina citri* reovirus (DcRV) | | Number of samples with *Diaphorina citri* picorna-like virus (DcPLV) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Adult | Nymph | Adult | Nymph | Adult | Nymph | Adult | Nymph | Adult | Nymph |
| **Region A** | 124 | 4 | 47 | 40 | 16 | 7 | 2 | 8 | 0 | 1 | 12 | 16 |
| **Region B** | 72 | 3 | 32 | 20 | 14 | 2 | 3 | 4 | 0 | 0 | 3 | 10 |
| **Region C** | 150 | 5 | 56 | 42 | 20 | 11 | 3 | 5 | 0 | 0 | 12 | 17 |
| **Region D** | 115 | 4 | 39 | 36 | 10 | 12 | 1 | 6 | 1 | 0 | 8 | 17 |
| **Region E** | 134 | 4 | 44 | 35 | 14 | 8 | 3 | 5 | 0 | 0 | 12 | 12 |
| **Region F** | 61 | 1 | 28 | 19 | 7 | 5 | 2 | 1 | 0 | 0 | 7 | 2 |
| | **656** | **21** | **Present in all regions (67% of samples)** | | **Present in all regions (19% of samples)** | | **Present in all regions (7% of samples)** | | **Present in 2 regions (<1% of samples)** | | **Present in all regions (20% of samples)** | |

*Each detection of the virus(es) shades the numbered box toward a dark green and represents a simple heat map of prevalence levels for each virus in each region, dark green representing higher levels and dark red representing lower levels.*

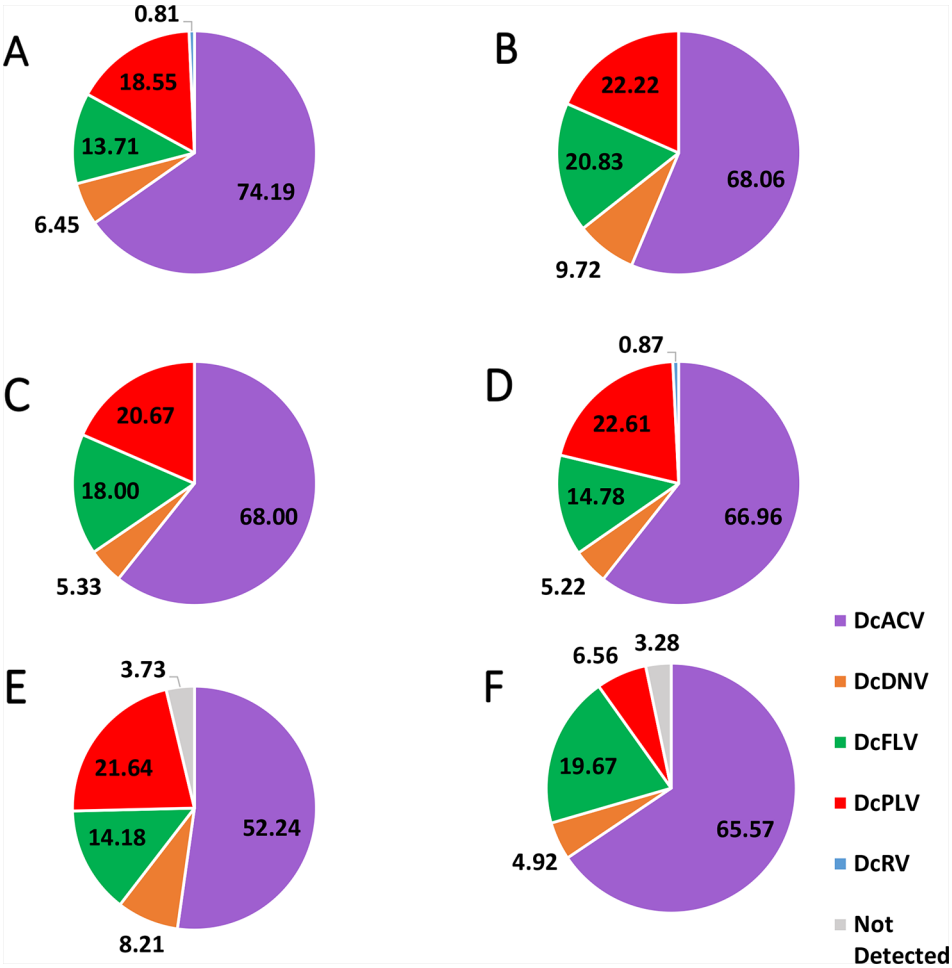


**FIGURE 2 |** Pie chart showing the percentage of each virus detected in **(A)** Florida Region A, **(B)** Florida Region B, **(C)** Florida Region C, **(D)** Florida Region D, **(E)** Florida Region E, and **(F)** Florida Region F. Note that in the majority of the Florida Regions, there were multiple samples that had multiple viruses; therefore, the majority of the percentages in the pie charts do not equal 100%. Means of the detected percentage of the viruses in each identified region were compared to each other and did not show significant difference (significance threshold set at $P < 0.05$).

The cDNA samples generated from RNA extracts during the study always tested positive for the ACP internal control, the ACP (*Dc*) wingless gene (data not shown). The ACP wingless gene was used for the confirmation of total RNA and cDNA integrities during this study. The detection of the wingless gene added further confidence in negative samples of the ACP-associated viral survey results.

## Verification of Novel Sequences From HTS Data in ACP Populations

HTS yielded between 24 and 35 million raw reads per cDNA library (data not shown). Subsequent analysis identified sequences of several diverse viruses in all ACP samples. As expected, some of these viral sequences shared high similarities with sequences of known ACP-associated viruses, including DcACV and DcFLV. However, there was also a substantial number of sequences sharing similarities with novel viruses that were not known to be present in the ACP (data not shown). For example, a novel virus sequence (~ 4.1 kb in length) with very low similarity (< 50%) to the RNA-dependent RNA polymerase (RdRp) gene of another reovirus from an insect in Africa (Hermanns et al., 2014) was discovered during our HTS analysis. To validate the authenticity and trace back the presence of this virus in our ACP populations collected from the various regions in Florida included in this study, we designed two sets of primers (**Table 2**): one targeting the 5' end of the predicted RdRp and another at the 3' end of the genomic RNA sequence. First, we screened and successfully detected this novel virus throughout the original county samples used for the initial composite sample using RT-PCR with these primers. Simultaneously, seasonal representative samples that were used for the screening of the five known ACP-associated viruses were also used for this novel virus to obtain a general representation of its prevalence (**Figure 3**). This virus, tentatively named

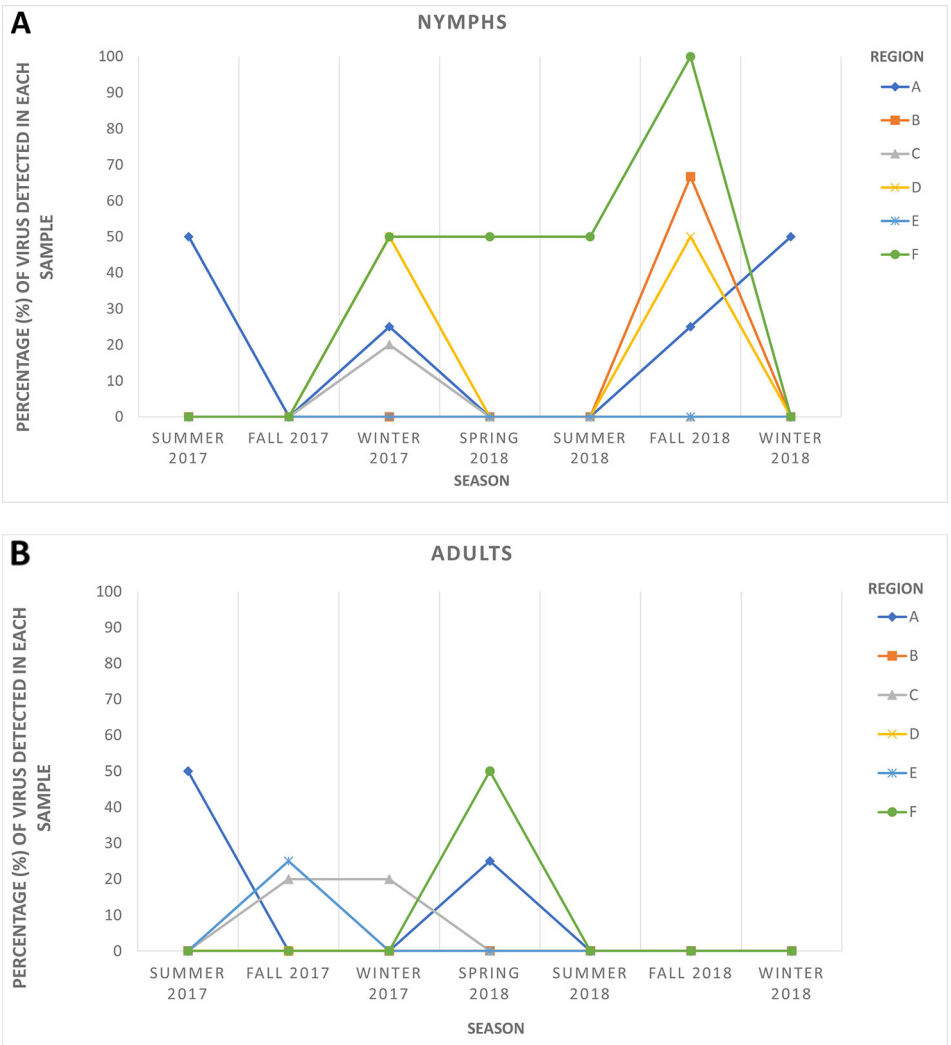


**FIGURE 3 |** The seasonal prevalence of the novel *Diaphorina citri* cimodo-like virus (DcCLV) in **(A)** nymphs and **(B)** adult ACPs over six seasons (one month per season) in the six larger Florida regions.

*Diaphorina citri* cimodo-like virus (DcCLV), was detected in both adult and nymph ACP populations. It was found in all of the six Florida Regions; however, it excluded most of the seasons in Region B, D, and E (**Figure 3**). Interestingly, we observed a higher incidence of this novel virus in nymph ACP samples than adults (**Figure 3**).

Furthermore, HTS analysis revealed the noticeably higher abundance of *Citrus tristeza virus* (CTV) sRNA sequences in all Florida regional ACP samples compared to any other known virus. More than 60% of all sRNA reads in each regional sample were matched to the CTV genome, with very few detectable reads matching to the DcACV genome (**Figure 4**). Only half of the regional samples had any DcFLV sRNA reads detected, and as expected, all of the samples had levels of unknown sRNA reads, which supports our hypothesis of novel viruses present in the ACP. Interestingly, an additional novel picornavirus, with 77% sequence similarity to *Graminella nigifrons virus* 1 (Chen et al., 2015), was also detected in Region A (data not shown). Yet, for all of the regions, CTV composed at least 60% of all HTS reads and suggests possible uptake of CTV by the ACP from infected trees during phloem-feeding.

## DISCUSSION

To achieve the goal of this study's first objective, we collected thousands of nymph and adult ACPs from 21 of Florida's major citrus-producing counties in order to effectively survey and monitor the diversity of viruses in the ACP microbiome (**Table 1** and **Figure 1**). Five previously identified ACP-associated viruses, DcACV, DcFLV, DcDNV, DcRV, and DcPLV were chosen from

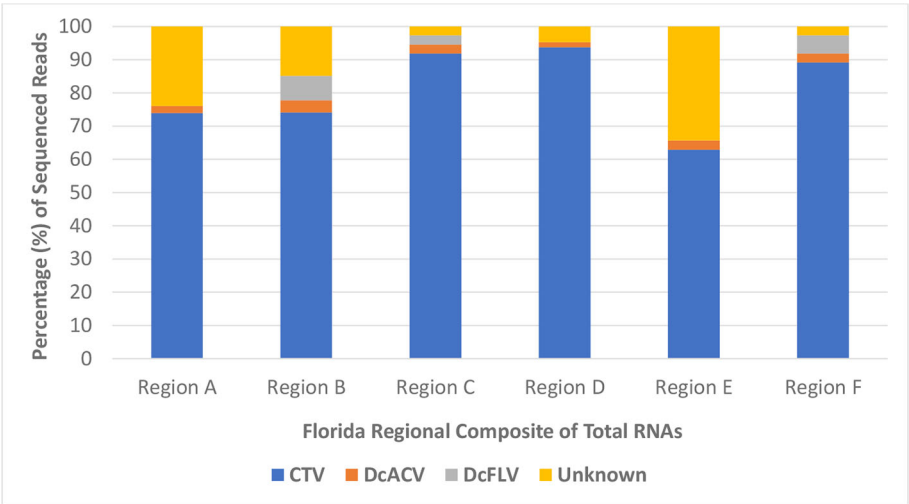


**FIGURE 4 |** The percentage of high throughput sequencing (HTS) reads per composite sample that matched to a known virus species or were categorized as unknown. CTV, *Citrus tristeza virus*; DcACV, *Diaphorina citri-associated C virus*; DcFLV, *Diaphorina citri flavi-like virus*.

prior literature investigating the ACP virome, applied to this survey study, and successfully detected and monitored in these populations over approximately eighteen consecutive months (six seasons). Although these viruses were previously found in ACPs from other countries, only three of them (DcACV, DcFLV, and DcRV) were reported in ACP populations from Florida (Marutani-Hert et al., 2009; Matsumura et al., 2016; Nouri et al., 2016a; Nouri et al., 2016b). However, our survey results indicate that all five viruses were widespread and present at varying incidences in ACP populations throughout Florida citrus groves (**Table 3** and **Figure 2**). To the best of our knowledge, this is the first study that has conducted a comprehensive and grove-based survey throughout Florida for establishing the prevalence levels of these viruses and the identification of two new viruses in ACP populations since their introduction into the state in 1998.

Our survey results indicate that the DcACV was detected at a higher level than any of the other four ACP-associated viruses in nymph and adult ACP populations collected throughout Florida citrus groves. And, DcACV RNAs were also detected in partially purified virions from nymph and adult ACPs (data not shown). This, along with the greater variance of DcACV incidence levels for all regions, shows the need for quantification of these viruses in future studies to compare how abundant they are in the ACP. DcFLV and DcPLV were also detected throughout most of the sampled citrus-producing counties, although not every month as was seen with DcACV, which suggests these viruses may exist at lower titers in the ACPs of these regions. Surprisingly, we also detected DcDNV throughout these vector populations, which has not been observed in Florida before this study (Nouri et al., 2016a). Remarkably, although Nouri et al. (2016a) did not detect the DNA virus DcDNV or the RNA virus DcPLV in their ACP samples from Florida, it was detected in ACPs throughout all Florida regions surveyed (**Table 3** and **Figure 2**). This finding supports the novel presence of this ACP-associated virus in Florida and adds increased diversity to the list of potential viral vectors to pursue for biological control. Finally, the extremely low incidence levels of DcRV detected in our study seemed to contrast with the >50% incidence level reported by Marutani-Hert et al. (2009). Even this information on the lack of viral detection in these collected ACP samples can strengthen the case of the diversity and fluctuation of the ACP virome within the same region of citrus groves sampled. Nonetheless, all five of the viruses in this study were detected in both adult and nymph ACP populations, which suggests an infectious and/or propagative nature of these viruses passing from adult female ACPs to nymphs.

RNAi defense mechanisms represent an integral part of the insect immune response to viral infections and allows for the substantial detection of viral sRNAs among total insect RNA extracted (El-Shesheny et al., 2013; Roossinck et al., 2015; Pecman et al., 2017). The insect vector of the HLB pathosystem, the ACP, also has these resistance capabilities, and was analyzed extensively during this study to further understand its virome as it relates to Florida citrus groves (Van Rij, 2011; Hall et al., 2013; Taning et al., 2016). Since the detection and prevalence of these five previously identified ACP-associated viruses allowed for only a piece of the ACP virome puzzle, we simultaneously compiled early ACP RNA samples of all county sites into six regional samples and subjected them to HTS technologies. Each of the six composite RNA samples were based on the combined regional counties (Region A-F) shown in **Figure 1**. When we applied HTS technologies toward analysis of these insects, the sequenced and analyzed results of the RNA samples revealed a frozen snapshot of the viral composition and identity of the Florida ACP virome, as shown in **Figure 4**. Although this analysis only represents a small subset of ACP populations in Florida citrus groves between August 2017 and May 2018, when combined with the results of our simultaneously

conducted PCR and RT-PCR survey screenings, it provides substantial evidence to the varying levels of viral prevalence detected. Not only does this further support the high prevalence of DcACV in Florida ACP populations, but also suggests that it might be well adapted to this insect host.

However, it should be noted that our survey results show the detection of each ACP-associated virus when originating from a five-insect subset specimen (composite sample) of each monthly-collected county sample. This consistent number of insects for each population was intended to standardize possible incidence levels detected of each virus. If subsets of ACPs used for RNA extraction and cDNA synthesis were increased, incidences of these viruses may change and show different prevalence levels.

The second objective of the current study was to examine populations of ACP from Florida citrus groves through HTS of small RNAs in an attempt to discover additional unknown viruses associated with this insect. Such metagenomic approaches with similar goals have been successfully implemented to discover diverse and novel viruses from field-collected mosquitoes, ACPs, *Drosophila* flies, and various other types of insects (Marutani-Hert et al., 2009; Ma et al., 2011; Cook et al., 2013; Coffey et al., 2014; Webster et al., 2015; Matsumura et al., 2016; Nouri et al., 2016a; Nouri et al., 2016b). A desirable translational outcome of this objective would be to identify ACP-associated viruses that have the potential to be used as biological agents to control *D. citri* and halt the spread of HLB, particularly in the endemic environment of Florida. In this study, we were able to identify and assemble nearly complete genome sequences of several putative novel viruses associated with ACP, including a novel reo-like virus and a picornavirus. To survey the newly identified reo-like virus, which is tentatively named *Diaphorina citri cimodo-like virus* (DcCLV), in six Florida regional ACP populations, specific primers were designed based on the fragments obtained from bioinformatics analysis and used to screen additional *D. citri* populations, which were not analyzed by HTS (**Table 2** and **Figure 3**). The DcCLV was detected in both adult and nymph ACP populations and was found in all six Florida regions, although at varying prevalence levels (**Figure 3**). However, we observed a higher incidence of this novel virus in nymph ACP samples than adults, which may suggest a plausible lethality to the ACP as infected nymphs may not survive to adulthood. Nevertheless, this hypothesis must be substantiated with more evidence gathered through further studies.

One of the unexpected outcomes of the HTS results was the discovery that CTV, a non-ACP-associated virus, is extremely abundant in the Florida ACP virome (**Figure 4**). To further validate this finding, we went back and screened representative cDNA samples of our ACP populations collected during early months of this study using RT-PCR with CTV-specific primers. We were able to detect this virus in both adult and nymph ACP samples collected throughout Florida citrus groves, as well as in subsequently purified additional virion samples from ACPs by immunogold labelling with CTV-specific antibodies using transmission electron microscopy (data not shown). Together these results cautiously suggest that although CTV is not causing noticeable decline or showing visible symptoms in citrus trees, the virus persists throughout Florida. The fact that non-pathogenic CTV-RNAi vectors are currently being deployed into the citrus host and our discovery of CTV persistence in Florida citrus groves calls for further scrutiny into possible cross-reactivity of viral strains (Hajeri et al., 2014). Unanswered questions regarding what CTV may be doing in the ACP warrants more studies. Future research into these molecular interactions may provide deeper insight into the uncertainty of CTV's modified deployment in the citrus host (Harper and Cowell, 2016). Furthermore, the detection of a novel picornavirus with high (77%) sequence similarities with *Graminella nigifrons virus 1*, a picornavirus known to be associated with leafhoppers (Chen et al., 2015), in our samples also deserves future inquiry into reasons for its presence in the ACP.

Comprehensive and grove-based viral surveys during this study (i.e., RT-PCR and PCR) and multiple sRNA read detections using HTS established that DcACV was the most prevalent and widespread ACP-associated virus in Florida citrus groves. Following the completion of this study, our specific approach for targeting the mortality of the ACP as a biological control will undoubtedly influence the choice of viral vector we will venture forth with. On the one hand, our research findings provide robust evidence for DcACV as the likely choice to pursue as a possible viral candidate in RNA interference technology. Its clear ubiquity deserves future research into possible utilization as an RNAi delivery machinery against the HLB vector. On the other hand, the low prevalence and detection of DcRV may be considered as unfavorable to the ACP and could serve as an even better viral candidate for RNAi delivery. Regardless of the virus' abundance, any modified viral vector will require high durability in the insect to avoid losing efficacy, as it will be constantly subjected to evolutionary changes and potential suppression by the ACP. Detection of all the viruses in both nymph and adult ACP samples suggest the potential of vertical transfer of each virus from parent to progeny and each virus' capability to remain in the ACP populations. DcACV's consistent detection and prevalence in ACPs and its prevalence in detection through HTS also suggests the highest stability observed amongst the viruses during this study in the ACP virome. DcACV may not be treated as a foreign entity if manipulated and re-inoculated into wild Florida ACP populations, which strengthens its potential as an RNAi vector tool for future studies.

In conclusion, this comprehensive survey and the successful detection of two novel and five previously characterized ACP-associated viruses, coupled with further HTS analysis of these Florida ACP populations, have provided innovative and clearer insight regarding the spatial and temporal prevalence of these viruses in natural grove conditions. These results have greatly increased our knowledge of a potential source of viral candidates to use against this economically devastating insect vector in Florida. To capitalize on this study, our next steps will include establishing the presence and significance of *C*Las in these same ACP RNA samples used for the ACP-associated viral surveys. We also hope to determine the precise tissue localization of these ACP-associated viruses in the ACP, which will help strengthen

future research by determining how efficient and effective this viral candidate's RNAi capabilities could be toward controlling the HLB disease cycle by killing the ACP.

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

## AUTHOR CONTRIBUTIONS

KB performed the experiments, collected ACPs, analysed the data and drafted the manuscript. SG helped with RT-PCRs and PCRs, ACP collections, statistical analysis, and editing the manuscript. MR completed all the HTS. OB and AL received the funds, designed and directed the study. OB identified grove sites, collected ACP, purified virions, analysed the data and wrote the manuscript. All authors reviewed and edited the manuscript.

## FUNDING

Funding for this project was provided by the Citrus Initiative Grant of University of Florida IFAS and U.S. Department of Agriculture (USDA) under grant number 2015-70016-23011.

## ACKNOWLEDGMENTS

We would like to thank Ana Redondo, Alec Pica, and George Ugartemendia for ACP collections, and the Florida citrus growers for allowing us entry into their groves for ACP collections. We would also like to thank Dr. Bryce Falk and his laboratory members for providing positive controls, and Minsook Hwang for technical assistance with HTS.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 14 October 2020
doi: 10.3389/fpls.2020.567212

# Effectiveness of the Brown Lacewing, *Sympherobius barberi* Banks as a Biological Control Agent of the Asian Citrus Psyllid *Diaphorina citri* Kuwayama

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The Asian citrus psyllid (ACP) *Diaphorina citri* Kuwayama is an economically important pest of citrus because it vectors the causal pathogens of huanglongbing (HLB) or citrus greening disease. Biological control is an important component of citrus pest management but requires consistent strengthening of its impact on pest complex. The brown lacewing *Sympherobius barberi* Banks is a known predator of several insect pests from Asia, Europe, and America. However, there is not much information about its effectiveness against *D. citri*. We evaluated *S. barberi* against the *D. citri* and frozen eggs of the Mediterranean flour moth *Ephestia kuehniella*, the latter is a common diet used for rearing predators in laboratories. Adult *S. barberi* successfully fed on *D. citri* eggs and nymphs under both light and dark conditions. *Diaphorina citri* was also suitable for the development and reproduction of *S. barberi* except for slightly prolonged larval development compared with *E. kuehniella* diet. The egg hatch from the total number of eggs laid on *D. citri* and *E. kuehniella* diets averaged 65% and 52%, respectively. Females laid 64% eggs on dimpled white paper compared to 36% combined on plain paper and leaves of citrus, orange jasmine, eggplant and cantaloupe. *Sympherobius barberi* released at densities of 2–6 adults against eggs and nymphs of *D. citri* on infested orange jasmine plants in the cages provided a reduction of 43–81% in the number of provided eggs or nymphs. In the field tests on *D. citri* infested citrus trees, reduction averaged 35% in five cohorts in which developing colonies of 28–32 nymphs were provided to one *S. barberi* per cage. Findings suggest the significant potential of *S. barberi* as a predator of *D. citri* and to contribute to reducing huanglongbing.

**Keywords: Asian citrus psyllid, citrus greening, biological control, predator, lacewings**

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***Edited by:***
*Marcos Antonio Machado, Instituto Agronômico de Campinas (IAC), Brazil*

***Reviewed by:***
*Niels Holst, Aarhus University, Denmark*
*Daniele Cornara, Consejo Superior de Investigaciones Científicas (CSIC), Spain*

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***Specialty section:***
*This article was submitted to Plant Pathogen Interactions, a section of the journal Frontiers in Plant Science*

***Received:*** *29 May 2020*
***Accepted:*** *18 September 2020*
***Published:*** *14 October 2020*

***Citation:***
*Khan AA, Afzal M, Stansly P and Qureshi JA (2020) Effectiveness of the Brown Lacewing, Sympherobius barberi Banks as a Biological Control Agent of the Asian Citrus Psyllid Diaphorina citri Kuwayama. Front. Plant Sci. 11:567212. doi: 10.3389/fpls.2020.567212*

## INTRODUCTION

The Asian citrus psyllid (ACP) *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) is a primary vector of the causal pathogens of huanglongbing (HLB) also known as citrus greening disease. It is an economically important pest of citrus in HLB affected regions (Catling, 1970). First described in Taiwan (Kuwayama, 1908), *D. citri* was later found in Punjab Pakistan and the rest of the region

(Hussain and Nath, 1927) and reported from Brazil in 1940 (Lima, 1947) and Florida in 1998 (Halbert, 1998). Huanglongbing in Florida was detected in 2005 (Halbert, 2005) 1 year after its identification from Brazil in 2004 (Texeira et al., 2005).

Several members of the order Neuroptera are known bio-control agents of multiple pests and are distributed all over the world. Chrysopidae and Hemerobiidae families received significant attention as bio-control agents because several species are voracious feeders of soft-bodied insects (Balduf, 1939; New, 1975).

Hemerobiidae family, with approximately 27 genera and 560 described species, includes many brown lacewings, which are predators of a wide range of insect pests (Oswald, 1993, 1994; New, 2001; Grimaldi and Engel, 2005). Most species of hemerobiids are predaceous in both imaginal and preimaginal stages (Miller and Cave, 1987). Adults of several species are generalist predators, and many are habitat-specific (Killington, 1937; Monserrat and Marín, 1996). Brown lacewings are known as natural enemies of the several insect pests in classical biological control (Sato and Takada, 2004). Both larvae and adults of several species are efficient predators on eggs and immature stages of several pests. Due to their prolonged longevity, high consumption rates, and high reproduction rate, they are established as important bio-control agents in the agro-ecosystem (MacLeod and Stange, 2005). *Sympherobius barberi* is native to Continental US, Canada and Mexico, while geographically it is distributed throughout the North America, Europe, and Northern Asia (excluding China), Middle America, Oceania and South America (Penny, 1977; Penny et al., 1997). Pacheco-Rueda et al. (2011) evaluated *S. barberi* against *Dactylopius opuntiae*. They reported a net reproductive rate ($R_o$) of 36.6, a daily intrinsic rate of increase ($r_m$) of 0.081, a generation time (T) of 44.27 days, and a finite reproductive rate ($\lambda$) of 1.084 for *S. barberi*.

Most soft-bodied insects and their eggs are preferred food for brown lacewing. Cutright (1923) observed the larva of the brown lacewing; *Micromus posticus* Walker consumes 41 aphids throughout its life. Yayla and Satar (2012) found out that larvae of *Sympherobius pygmaeus* Rambur on the diet of *Planococcus citri* developed in 31 days at 25°C and female laid maximum of 258 eggs.

Our objectives for these studies were (1) to investigate the development, reproduction and predation potential of *S. barberi* on the diet of *D. citri*, in comparison with frozen eggs of the Mediterranean flour moth *Ephestia kuehniella* (Lepidoptera: Pyralidae), and (2) to evaluate *S. barberi* for reducing *D. citri* populations under greenhouse and field conditions. Eggs of *E. kuehniella* is a conventional diet for rearing several predatory species. The findings of this study may improve our understanding of utilizing *S. barberi* for augmentative biological control purposes in citrus crops worldwide, particularly in systems where increased chemical control is negatively impacting naturally occurring predators.

# MATERIALS AND METHODS

## Study Location, Insects, and Experimental Conditions

Colonies of *D. citri* and *S. barberi* were maintained and experiments conducted at the Southwest Florida Research and Education Center (SWFREC) of the University of Florida-IFAS, Gainesville, FL, United States. *Diaphorina citri* colony was maintained on orange jasmine *Murraya paniculata* (L.), in a climate-controlled glasshouse set at 28°C. *Murraya paniculata* is a close relative of citrus, and one of the preferred hosts of *D. citri*. *Sympherobius barberi* adults originally obtained from Foothill Agricultural Research, Inc. (550 W Foothill Pkwy, Corona, CA, United States) were used to maintain its colony on frozen eggs of *E. kuehniella* (Koppert Biological Systems, Romulus, MI, United States) and nectar honey. The colony used several groups of 20 adults per 3-L ventilated plastic jar. The frozen eggs of *E. kuehniella* and nectar honey were provided for food and a small cube of moist sponge for water. Shoots of *M. paniculata* and pieces of paper towel were provided as a substrate for oviposition. Eggs laid on paper towel or leaves were then placed in 20 ml (5-DRAM) snap cap vials and observed for hatching. The colony was maintained in an incubator (Percival, Model I36LLC8, Percival Scientific, Inc., Perry, IA, United States) set to a photoperiod of 16:8 (L:D) at 25°C. Upon hatching, neonates were collected daily, and experiments conducted in the incubator and conditions described for the colony.

## Laboratory Experiments

### Feeding

Twenty-four hours after emergence, adult *S. barberi* were used to test for feeding on *D. citri* nymphs under light and dark conditions in the growth chamber. Sixty adults of *S. barberi* were distributed randomly across three different diets (1) psyllid eggs, (2) psyllid nymphs $2^{nd}$ and $3^{rd}$ (small) instars, and (3) psyllid nymphs $4^{th}$ and $5^{th}$ (large) instars. One adult was provided with 25–30 psyllid eggs and 20 small or large nymphs on young orange jasmine shoots in the petri dish (9-cm diameter and 1.5-cm height). The petri dishes were covered with perforated parafilm to prevent the escape of nymphs and provide ventilation. The number of eggs and nymphs consumed by *S. barberi* were recorded at 6, 12, and 24 h.

### Reproduction

Male and female were distinguished by examining their genital appendages using the 14X Triplet Hasting Magnification hand lens. The male has flattened shaped terminalia with two claws, while the female has tipped end with two knobs (**Figures 1A,B**). Each pair of male and female was held in 100 ml perforated snap-cap plastic vial arena and provided with diets under three treatments to evaluate reproduction; (1) nectar honey on a paper strip (2) frozen eggs of *E. kuehniella* and (3) eggs and younger nymphs of *D. citri* on shoots of *M. paniculata*. Shoots of *M. paniculata* and dimpled white paper towel (1"X4")

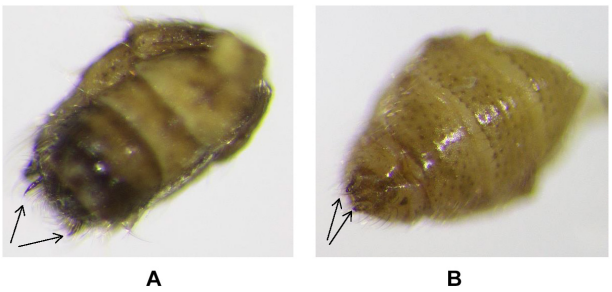


**FIGURE 1 |** Abdominal terminallia of *S. barberi,* Male: **(A)** and Female: **(B)**. Male and female pairs for reproduction tests were separated by distinguishing genital appendages morphologically, using 14X triplet hasting magnification hand lens. Male **(A)** has flattened shaped terminalia with two claws, while Female **(B)** has tipped end with two knobs. Slide photos were taken by using Olympus SZX16 stereo microscope fixed with DP21 digital camera.

**FIGURE 2 |** Dissected *S. barberi* female with egg cluster. Female was dissected under microscope (using fine needle/forceps) for the presence of egg cluster before and after the experiment. Female and cluster are visible in the figure. This dissection was also done to confirm the morphological differences in the male and female genital appendages. Slide photo was taken by using an Olympus SZX16 stereo microscope fixed with digital camera DP21.

were provided in the vials as a substrate for oviposition. Eggs laid by *S. barberi* were collected for 3 weeks, and food replaced every 24 h.

Eggs from each male-female pair were kept in 20 ml (5-DRAM) snap cap vials in the growth chamber under conditions described above and examined daily for hatching. Upon death, females were dissected and examined under a stereomicroscope (Olympus SZX16, SDF-PLAPO, Japan) fixed with a digital camera (DP21) to check for the presence of eggs (**Figure 2**). A separate experiment was conducted to test female preference to lay eggs on six different substrates including, dimpled white paper, black paper, and leaves of four different plant species, including Citrus, Orange jasmine, Eggplant, and Cantaloupe using 10 female replicates.

### Development

Eggs obtained from the parents maintained on the diets of nectar honey, frozen eggs of *E. kuehniella*, and eggs and nymphs of *D. citri* were held separately for each diet at one egg per 20 ml (5-DRAM) snap cap vial. There were 10 replicates for each of the three diets. Upon hatching, larvae were reared on the same three diets on which their parents were maintained. Food was replaced daily and data recorded on larval survival, pupation, and adult emergence. The longevity of adult *S. barberi* was evaluated in two experiments (1) including diets of eggs of *E. kuehniella*, eggs of *D. citri*, and nymphs of *D. citri*, and (2) mixed diets including eggs plus nymphs of *D. citri* as a separate treatment, with honey and honey by itself. Twenty replicates were performed for each diet.

## Greenhouse Experiments

The potted plants of *M. paniculata* were pruned and placed individually in bugdorm cages to induce new shoots needed for psyllid reproduction and development. The bugdorm cage (58 cm × 58 cm × 66 cm) made from clear plastic included two side panels of fine Polyester netting for ventilation. Psyllid adults used in the experiment were obtained from a colony maintained on *M. paniculata*, in a glasshouse at 25 ± 2°C provided with 1000W metal-halide light set to 16: 8 (L:D). The adult psyllids of mixed gender (50 males, 50 females) were released in each experimental cage.

The plants were checked daily for oviposition by psyllids. Sixteen plants adjusted to an average of 100 eggs on 3–4 shoots/plant were moved to new cages at one per cage. The plants were assigned randomly to three release densities of *S. barberi* adults (2, 4, and 6 adults/cage) and control without any adult for a total of four replicates per predator density treatment. Eggs were counted on the plants 3 days after adult release and before hatch

**TABLE 1 |** Number (Mean ± SE) of eggs and small or large nymphs of *Diaphorina citri* consumed by adult *Sympherobius barberi* during 24 h under light or dark conditions.

| Diet | 6 h | | 12 h | | 24 h | |
|---|---|---|---|---|---|---|
| | Light | Dark | Light | Dark | Light | Dark |
| Eggs | 8.10 ± 2.27 a | 7.60 ± 1.56 a | 15.90 ± 2.42 a | 9.70 ± 1.72 b | 20.00 ± 3.20 a | 19.40 ± 1.34 a |
| Small nymphs | 19.50 ± 0.27 a | 19.00 ± 0.26 a | 19.90 ± 0.10 a | 20.00 ± 0.00 a | 20.00 ± 0.00 a | 20.00 ± 0.00 a |
| Large nymphs | 12.00 ± 2.21 a | 14.10 ± 1.13 a | 17.70 ± 1.20 a | 16.60 ± 0.95 a | 19.00 ± 0.98 a | 19.00 ± 0.60 a |

*Forty-eight-hour old adults received from a commercial source were reared on nectar honey and starved for 24 h prior to the test. Means within rows followed by the same letters are not significantly different between light and dark conditions at a particular time (P > 0.05).*

**TABLE 2 |** Development time (Mean ± SE) of *Sympherobius barberi* on diets of *Diaphorina citri, Ephestia kuehniella*, and nectar honey.

| Diet | Egg stage (days) | Larval stage (days) | Pupal stage (days) |
|---|---|---|---|
| *E. kuehniella* | 5.00 ± 0.09 a | 12.40 ± 0.27 b | 9.80 ± 0.61 a |
| *D. citri* | 5.04 ± 0.09 a | 15.70 ± 0.58 c | 11.43 ± 1.43 a |
| Nectar honey | 5.00 ± 1.00 a | 10.30 ± 0.40 a | –– |

*Means in columns followed by the same letters are not significantly different (P > 0.05).*

to assess the number consumed by the predator. The plants were kept in the same cages to evaluate *S. barberi* predation on nymphs that emerged from the viable eggs. Nymphs consumed, dead, or emerged to adults were counted at an interval of 1 day for 1 week.

## Field Experiment

Four years old 5 ft high 'Hamlin' citrus plants at Southwest Florida Research and Education Center, Immokalee, FL, United States were pruned to encourage the development of young shoots needed for psyllid reproduction and development. Once infested with *D. citri*, 20 shoots were assigned randomly to two groups designated for release of *S. barberi* and control with no release. The number of psyllid nymphs (mostly younger instars $2^{nd}$ and $3^{rd}$) were counted on each shoot using the14X Triplet Hasting Magnification hand lens. Shoots were enclosed with sleeve cages made from fine mesh organdy. Those assigned for the predator's release received one adult *S. barberi* per shoot with a colony averaging 28–32 nymphs. Data regarding the consumption of psyllid nymphs and the emergence of adult psyllids in all cages were recorded at an interval of 1 day. *S. barberi* were moved to new shoots within the same age range of *D. citri* nymphs every 2–3 days until death. The previously used shoots were brought to the laboratory, and *D. citri* nymphs and adults were counted. In total, 42 nymphal colonies were used in the five cohorts, the first three cohorts with 10 nymphal colonies in each and $4^{th}$ and $5^{th}$ cohort with 7 and 5 colonies, respectively. All nymphs in the control colonies ($n$ = 10) were allowed to develop to adulthood and counted in the laboratory.

## Statistical Analysis

A generalized linear model (GLM) procedure was used for Analysis of Variance (ANOVA) followed by Fisher's least significant difference (LSD) test at $P = 0.05$ to discern differences in number of eggs and nymphs consumed under light and dark conditions. Data on larval survival to pupation and adult eclosion were analyzed by using the GLIMMIXMACRO model with a logit link function to transform data (SAS Institute, 2012). The data for egg and larval development time (days), fecundity, fertility (percent eggs hatched and the number of larvae per female), adult longevity, nymphal consumption by predators in the greenhouse and field experiments were analyzed by PROC GLM and treatment means separated using LSD.

# RESULTS

## Feeding of *S. barberi* on Eggs and Nymphs of *D. citri*

There was no significant difference in the consumption of eggs and nymphs by *S. barberi* between light and dark condition ($P > 0.05$) except for eggs at 12 h ($F_1 = 4.35$, $P = 0.05$) when consumption rate averaged 15.90 ± 2.4 in light and 9.70 ± 1.7 in the dark (**Table 1**).

## Development of *S. barberi* on *D. citri* and *E. kuehniella* Diets

The development time of the eggs from parents reared on the diets of *E. kuehniella, D. citri,* and nectar honey did not differ and averaged 5 days among the three diets ($P > 0.05$, **Table 2**). Larvae reared on the same three diets differed significantly in their development time to pupation ($F_{2,27} = 39.54$, $P < 0.001$, **Table 2**). Development time was prolonged on the diet of *D. citri* (15.70 ± 0.58 days), followed by the diets of *E. kuehniella* (12.40 ± 0.27 days) and nectar honey (10.30 ± 0.40 days). Larval survival to pupation was 100% on the diet of *E. kuehniella* and 70% on *D. citri* and none from those on nectar honey pupated. Pupal stage lasted 11.43 ± 0.43 days for those on the diet of *D. citri* and 9.80 ± 0.61 days for those on *E. kuehniella* and did not differ statistically ($P > 0.05$, **Table 2**). Adult emergence from the pupae was 100% on the diets of *D. citri* and *E. kuehniella*. There was no significant difference in adult longevity when tested on the diets of eggs of *E. kuehniella* and eggs or nymphs of *D. citri* ($P > 0.05$, **Table 3**). The provision of honey with the diet of *D. citri* did not improve adult longevity ($P > 0.05$, **Table 3**).

In the experiment to test female oviposition preference for substrate, 64% of total eggs were laid on dimpled white paper and 36% on the remaining five substrates which included black paper, orange jasmine leaves, citrus leaves, eggplant leaves and

**TABLE 3 |** Longevity (Mean ± SE) of adult *Sympherobius barberi* on diets of *Diaphorina citri, Ephestia kuehniella* and nectar honey alone or mixed.

| Experiment 1 | | Experiment 2 | |
|---|---|---|---|
| **Diet** | **Longevity (days)** | **Diet** | **Longevity (days)** |
| *D. citri* nymphs | 18.40 ± 0.964 a | Nectar honey | 16.00 ± 1.168 a |
| *D. citri* eggs | 19.15 ± 1.230 a | Honey + *D. citri* (eggs + nymphs) | 15.95 ± 1.243 a |
| *E. kuehniella* eggs | 21.45 ± 1.276 a | *D. citri* (eggs + nymphs) | 12.65 ± 1.240 a |

*Forty-eight-hour old adults received honey on a paper strip, eggs of E. kuehniella on the surface of the petridish, and eggs or nymphs of D. citri on the shoot of M. paniculata. Means with the same letters within a column are not significantly different (P > 0.05).*

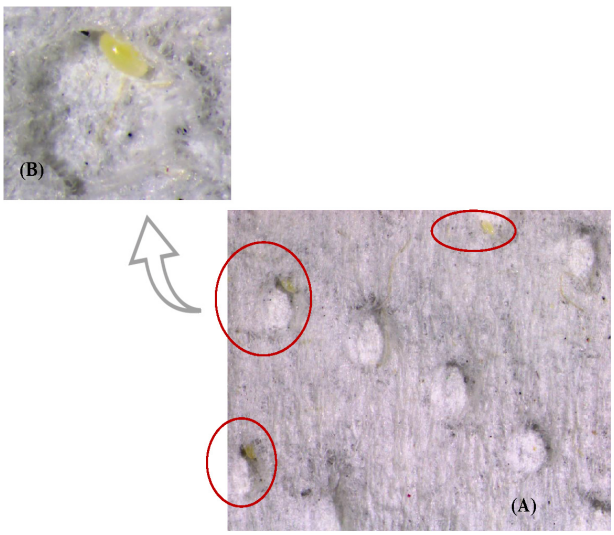


**FIGURE 3 | (A)** Eggs laid by *S. barberi* on dimpled white paper. Slide photo was taken by using an Olympus SZX16 stereo microscope fixed with a DPI-21 digital camera. **(B)** Magnified view of a single dimple with egg.

cantaloupe leaves (**Table 5** and **Figures 3A,B**). An average of 27 eggs per female were laid during the observation time of 1 week.

## Reproduction of *S. barberi* on *D. citri* and *E. kuehniella* Diets

An average of 25–30 eggs laid per female did not differ significantly between the diets of *D. citri* and *E. kuehniella*; however, were significantly more than few laid on honey ($F_{2,42}$ = 12.522, $P < 0.001$, **Table 4**). A significantly higher percentage of eggs hatched on the diet of *D. citri* (65%) compared to *E. kuehniella* (52%) (**Table 4**). There was no significant difference in the number of larvae per female ($P > 0.05$, **Table 4**).

## Effect of *S. barberi* Predation on *D. citri* in Potted Orange Jasmine Plants in the Greenhouse and on Citrus Trees in the Field

The number of *D. citri* eggs on potted orange jasmine plants were significantly reduced in the cages where *S. barberi* were released compared to no release control cages ($F_{3,15}$ = 23.31, $P$ = 0.0001, **Figure 4**). Most reduction of 69% was observed in the treatment where six adults were released per cage, followed by 50 and 43% in the treatments with four and two adults per cage, respectively. Significant reduction in nymphs was also observed with the release of *S. barberi* on the potted orange jasmine plants in the cages compared with control ($F_{3,15}$ = 16.45, $P$ = 0.0005, **Figure 4**). Most reduction of 81% at high release density did not differ from 74% at medium release density, although both these reductions were significantly more than 43% observed at low release density. There was a trend of increased reduction in *D. citri* with an increase in the rate of *S. barberi* release. Under field conditions, a significant average reduction of 35% was observed in the nymphal colonies of *D. citri* caged with *S. barberi* on citrus trees compared with only 4% in control ($F_{1,9}$ = 29.43, $P$ = 0.0004). *Diaphorina citri* reduction averaged between 25 and 42% (**Figure 5**), over five cohorts, which included 42 nymphal colonies and each with one *S. barberi*.

# DISCUSSION

This first detailed investigation of *S. barberi* predation against *D. citri* demonstrates the aggressive feeding behavior of this predator on psyllid eggs and nymphs. *Sympherobius barberi* were effective in consuming all the three life stages including, eggs, small nymphs, and large nymphs of *D. citri* under both light and dark conditions. There was a gradual increase in consumption rate over time, which was more evident for eggs (40 to 95% from 6 to 24 h) and large nymphs (60 to 95% from 6 to 24 h), while 95% of the small nymphs were consumed within 6 h. The feeding on eggs was initially low and improved over time, probably due to the concealed locations where they were laid, such as newly developing unfolded leaves and predator ability to reach into those locations. For large size predators such as lacewings and ladybeetles, it is difficult to attack prey in such locations, and therefore smaller predators such as predatory mites are more useful to attack eggs and neonates in such locations (Juan-Blasco et al., 2012). The difference of 35% in the consumption rate on small and large nymphs observed at 6 h was reduced to 5–10% within 12–24 h, suggesting that predator will most likely be negatively impacting significant nymphal populations encountered under field conditions. The predator preference of prey type or stage is reported for other lacewing species and pests and could impact predation rates in the field. For example, when all instars of pea aphid were presented in equal numbers to Tasmanian lacewing *Micromus tasmaniae* adults, they killed 74–90% of the first instars, 9–26% of second instars but none of the instar third and fourth (Leathwick, 1989). Islam and Chapman (2001) observed predation rate of 10

**TABLE 4 |** Fecundity and fertility (Mean ± SE) of *Sympherobius barberi* on diets of *Diaphorina citri, Ephestia kuehniella* and nectar honey over 3 weeks.

| Diet | Fecundity (no. of eggs/female) | Fertility (% eggs hatched) | Fertility (no. of larvae/female) |
|---|---|---|---|
| *E. kuehniella* | 30.13 ± 7.18 b | 51.64 ± 6.03 a | 13.20 ± 3.07 a |
| *D. citri* | 25.47 ± 2.84 b | 64.96 ± 6.07 b | 14.67 ± 1.19 a |
| Nectar Honey | 0.27 ± 0.15 a | —- | —- |

*Forty-eight-hour old adults were separated by distinguishing their genital characters to establish male and female pairs. Means with the same letters within a column are not significantly different (P > 0.05).*

**TABLE 5 |** Percent of eggs laid on six different substrates by *S. barberi* provided with the mixed diet of *D. citri*, nectar honey and *E. kuehniella*.

| No. | Substrate | Percent eggs laid |
|---|---|---|
| 1 | Dimpled white paper | 64 |
| 2 | Black paper | 11 |
| 3 | Cantaloupe leaves | 9 |
| 4 | Orange jasmine leaves | 7 |
| 5 | Eggplant leaves | 5 |
| 6 | Citrus leaves | 4 |

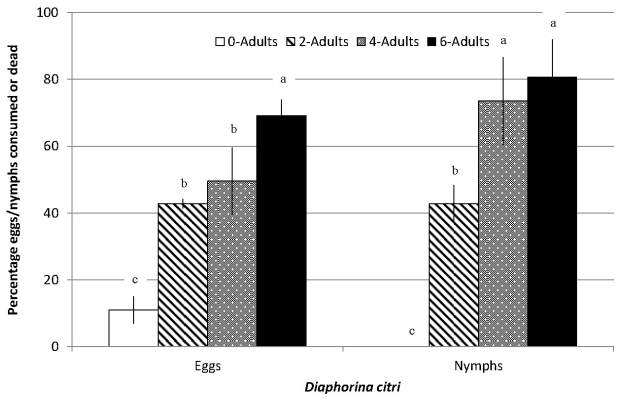


**FIGURE 4 |** Impact of different release rates of *S. barberi* adults on eggs/nymphal colonies of *D. citri* developing on orange jasmine plants caged in the green house. Significant reduction in *D. citri*, averaging 43–81% across three release rates compared to zero-10% in control. Columns sharing the same letter within eggs or nymphs are not significantly different ($P > 0.05$).

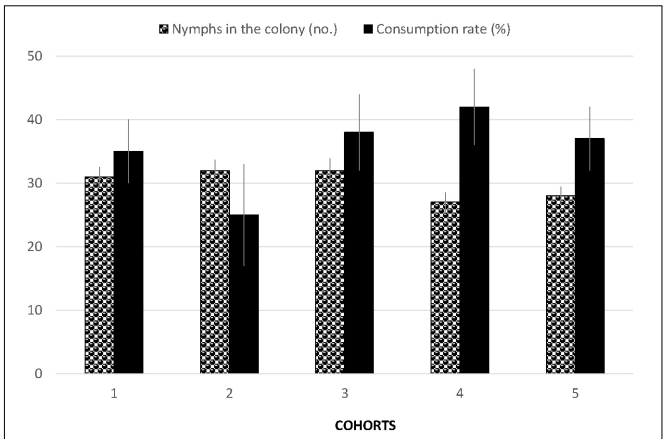


**FIGURE 5 |** Mean number of *D. citri* nymphs in the colonies (Black and white columns) and percentage consumed (Black columns) by *S. barberi* in five cohorts. The first three cohorts contained 10 colonies each followed by 7 and 5 colonies in the 4$^{th}$ and 5$^{th}$ cohort, respectively. Each cohort lasted 2–3 days when the same *S. barberi* were shifted to new colonies.

*Brevicoryne brassicae* aphids per day by the larval stage of brown lacewing *Micromus tasmaniae*, which is half of the predation rate of adult *S. barberi* on *D. citri* nymphs in the present study, suggesting better prey suitability for the predator in the latter scenario. However, the consumption rate by larvae of *S. fallax* on nymphal stages of a mealybug, *Pseudococcus longispinus,* was higher in another study (Gillani et al., 2009). We tested *S. barberi* against *D. citri* in two age groups. The successful feeding on young and mature nymphs containing 2–3 instar and 4–5 instar, respectively, followed by the experiment showing successful larval development in a diet of mixed instars indicate that they will be able to feed across all instars. Additionally, the developing colonies of *D. citri* nymphs in the field contain multiple instars which provide *S. barberi* enough choices to reduce pest population. In some situations, citrus shoots get colonized by more than one pest such as aphids and psyllids, and therefore, testing prey preference between species will be another interesting subject for a future investigation.

Larval survival of *S. barberi* to pupation on the diet of *D. citri* nymphs averaged 70% compared with 100% on the diet of frozen eggs of *E. kuehniella*. The larval development prolonged on the diet of *D. citri* nymphs compared to *E. kuehniella* eggs. The *Ephestia* eggs is a typical diet used for commercial rearing of several predators, which support maximum survival in most cases (Qureshi and Stansly, 2011; Khan et al., 2016), whereas *D. citri* was a new prey for the *S. barberi.* The presence of wing buds in the late instars nymphs of *D. citri* may have caused some deterrence for the *S. barberi* larvae and reduced the consumable body contents resulting in predator spending more time and energy in handling nymphs thus prolonged development and reduced survival in some cases. However, the pupation time of *S. barberi* did not differ between the diets of *D. citri* and *E. kuehniella,* and survival was 100% on both diets. The total development time from egg hatch to adult emergence was 26–28 days for *S. barberi* in the present study. Pacheco-Rueda et al. (2011) reported similar development time for this predator on the diet of *Dactylopius opuntiae*. Lara and Perioto (2003) reported that several species of hemerobiids complete their life cycle in 23–40 days at 23°C, and Vargas (2007) stated 23 days life cycle for *Chrysoperla carnea* at 26°. The survival rate of 70% on *D. citri* in the present study was better than some much lower rates reported for other Hemerobiids such as 2–25% for *M. tasmaniae* (Syrett and Penman, 1981), and 30% for *Hemerobius pacificus* (Neuenschwander, 1975) which most likely were impacted by rearing conditions including prey, condensation, and cannibalism. When reared individually, as in the present study, the survival of *M. tasmaniae* was in the range of 83–100% (Leathwick, 1989).

Pacheco-Rueda et al. (2011) observed an average adult longevity of 39 days for *S. barberi* on the diet of *Dactylopius opuntiae* and average of 1.89 eggs per day per female. In the present study, *S. barberi* adults evaluated in the two experiments lived between 13 and 19 days, on the diet of *D. citri*, and female produced an average of 25 eggs during a 3-week period, the latter not very different from the previous study. The difference in longevity may be due to the differences in prey and experiment conditions. The 65% of the eggs produced by *S. barberi* on the *D. citri* diet were fertile, suggesting suitability of psyllid for the successful reproductive performance of this predator.

*Sympherobius barberi* negatively impacted *D. citri* by providing significant reduction in the psyllid populations on

infested orange jasmine plants under semi-natural conditions in the screenhouse and on infested citrus trees in the field. Reduction of 43–81% in the eggs and nymphs was observed when *S. barberi* were released at the rate of 2–6 adults with 100 or fewer eggs or nymphs of *D. citri* per cage. There also was a trend of increased reduction in *D. citri* with an increase in the release rate of *S. barberi*. In the field conditions, one adult caged with an average of 28–32 nymphs provided a reduction of 35%. The evidence of *S. barberi* feeding, development, and reproduction on *D. citri* and negative impact on psyllid populations under semi-natural conditions suggest that this species may play a significant role as a predator of *D. citri*. However, open releases and evaluations are needed in the groves. Legaspi et al. (1996) investigated the field release of *Chrysoperla rufilabris* in the large field cages in watermelon fields using densities of 10, 25, and 50 larvae per cage. They reported approximately 35% more whiteflies in control with no release over the entire season as compared to the predator treatment with the highest whitefly counts. Similarly, Daane et al. (1993) observed a 35% reduction of leafhopper *Erythroneura variabilis* in the vineyard fields with the release of different species of lacewings. *Sympherobius barberi* like other lacewings attacks many pests, some of which such as whiteflies, aphids etc are found in multiple crops and may help support the establishment of this predator.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

## FUNDING

We are thankful to the Higher Education Commission of Pakistan, for funding the Ph.D. research work of AK, part of which including work of this investigation was conducted at the University of Florida SWFREC, Gainesville, FL, United States.

## ACKNOWLEDGMENTS

We thank Foothill Agricultural Research, Inc. (550 W Foothill Pkwy, Corona, CA, United States) for providing the initial shipments of *Sympherobius barberi*.

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